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SPHERIX

November 19, 2008

Office of Food Additive Safety
HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

NOV 20 2008

To Whom It May Concern:

Enclosed please find three copies of "Generally Recognized As Safe (GRAS) Notification for the Use of *Bifidobacterium longum* BB536 in Selected Foods". This GRAS notification has been prepared by Spherix Incorporated for Morinaga Milk Industry Co., Ltd.

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., CEO, Spherix Incorporated, 6430 Rockledge Drive, Suite 503, Bethesda, Maryland, 20817, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ckruger@spherix.com.

Should you have any questions or concerns, please contact me at the number listed above.

Sincerely,

Claire L, Kruger, Ph.D., D.A.B.T.
Chief Executive Officer

Enclosures:

Original and 2 copies of "Generally Recognized As Safe (GRAS) Notification for the Use of *Bifidobacterium longum* BB536 in Selected Foods"

Original and 2 copies of the GRAS Panel Consensus Statement for the above-referenced GRAS Notification

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Generally Recognized As Safe (GRAS) Notification for the Use of *Bifidobacterium longum* BB536 in Selected Foods

Prepared for:

Morinaga Milk Industry Co., Ltd.
Tokyo, Japan

Prepared by:
Spherix Incorporated
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August 1, 2008

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I. GRAS EXEMPTION CLAIM

A. NAME AND ADDRESS OF SPONSOR

Morinaga Milk Industry Co, Ltd.
33-1, Shiba 5-Chome, Minato-ku
Tokyo 108-8384, Japan
Tel: 81-3-3798-0152
Fax: 81-3-3798-0107
interntl@morinagamilk.co.jp

B. COMMON OR USUAL NAME OF GRAS SUBSTANCE

The substance that is the subject of this Generally Recognized As Safe (GRAS) determination is *Bifidobacterium longum* BB536 *(B. longum* BB536). *B. longum* BB536 is a strain of the species *Bifidobacterium longum.* The bacterium has been deposited with the American Type Culture Collection (ATCC) and is designated BAA-999^{TM}. *B. longum* BB536 cultures are used by Morinaga Milk Industry Co, Ltd. to manufacturer products sold under the names of FC-B, FCB-M1, BIFILON-50F, BIFILON-50N, BIFILON-50T, BIFILON-EX, and BIFILON-EX/LM.

C. INTENDED USE

B. longum BB536 is intended to be added to a variety of foods. The food categories to which *B. longum* BB536 will be added are listed in Table I-1. The proposed maximum use level of *B. longum* BB536 in each food category is 1 x 10^{10} CFU *B. longum* BB536 per serving. Serving sizes for the intended use categories correspond to the Reference Amounts Customarily Consumed (RACC) (21 CFR 101.12).

Table I-1. Food Categories Proposed for Addition of *B. longum* BB536

Breads/baked goods	
	bars; includes meal replacements, high protein, snack bars
	biscuits
	breads/rolls (yeast); includes bagels, croissants, English muffins, pizza crust
	breakfast pastries; includes Danish
	cakes, includes coffee cakes
	cobblers, turnovers, strudels, crisps
	cookies/bars
	crackers
	doughnuts
	pies
	quick breads; includes breads, muffins, popovers, cornbread
Cereals	
	breakfast cereals, cooked; includes grits, oatmeal, cream of wheat, wheat cereal
	breakfast cereals, ready-to-eat
Dairy products/dairy-based foods and dairy substitutes	
	cheese spreads
	cheese, imitation
	cheese, processed
	cream substitutes
	cream, heavy
	fermented milk; includes buttermilk and kefir
	flavored milk beverage mixes
	frozen desserts; includes ice cream, ice milk, frozen yogurt, frozen novelties, milk shakes
	follow-on infant formula
	imitation milk
	infant follow-on formula
	meal replacements, liquids and dry mixes
	milk, plain and flavored; includes cocoa, chocolate milk, fruit milks, coffee drinks (fluid/dry)
	puddings and custards
	smoothies
	whipped toppings
	yogurt
Fruit Products	
	juices and nectars; includes citrus, non-citrus, vegetable and blends
	frozen fruit
	frozen juice bars, ices
Miscellaneous	
	candies; includes hard candies, mints, chocolate, and all other types of confections
	chewing gum
	cocoa powder
	condiment sauces, including catsup, BBQ, taco, steak, cocktail, Worcestershire, teriyaki, tartar
	flavored beverage syrups
	fruit flavored powder beverage mixes
	gelatin desserts, plain or with fruit
	gravies
	margarine
	peanut and other nut butters/spreads
	snack foods; including chips, popcorn mixtures
	weaning foods (for children 12 months of age and older; includes dry cereal, snacks, juices)

D. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of *B. longum* BB536 as an ingredient in foods at a maximum level of 1 x 10^{10} CFU *B. longum* BB536 per serving at the end of product shelf-life as described in Section C of this chapter is based upon scientific procedures as described under 21 CFR §170.30(b). The intake of *B. longum* BB536 from the intended uses specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). To demonstrate that *B. longum* BB536 is safe, and GRAS, under the intended conditions of use, the safety of the intake of *B. longum* BB536 has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of *B. longum* BB536 as an ingredient in foods has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1) Bifidobacteria are naturally occurring bacteria that contribute to the composition of the gut microflora of humans. Bifidobacteria represent up to 25% of the cultivatable fecal bacteria in adults and 80% in infants, and *Bifidobacterium longum* has been detected in feces from infants and adults.

2) *Bifidobacterium longum* BB536, a strain of *B. longum,* is a Gram-positive anaerobic bacterium. *B. longum* BB536 was originally isolated from a healthy infant in 1969. The bacterium has been deposited with the American Type Culture Collection (ATCC) and is designated BAA-999™.

3) The maintenance of the original frozen culture has been tightly controlled to ensure purity and stability of the strain.

4) Finished products made with *B. longum* BB536 cultures reproducibly meet compositional standards and comply with limits on contaminants appropriate for food-grade ingredients. Product specifications are set to assure that *B. longum* BB536 is suitable for use in food.

5) Bifidobacteria are commonly consumed in fermented foods throughout the world. *B. longum* BB536 was first commercially available in Japan in 1977 and the availability of *B. longum* BB536 on the European market began in 1986.

6) *B. longum* BB536 has been tested for parameters outlined in the Food and Agriculture Organization of the United Nations/World Health Organization's (FAO/WHO) guidelines for the evaluation for microbes for probiotic use in foods. Results from these tests

provide evidence that *B. longum* BB536 is safe for use in foods, namely:

- Available antibiotic resistance pattern suggests that *B. longum* BB536 does not present concerns for antibiotic resistance in humans.
- *B. longum* BB536 produces predominantly L-lactic acid, while production of D-lactic acid is negligible.
- *B. longum* BB536 has been reported to deconjugate bile salts. The production of deconjugated bile salts was concurrent with bacterial growth, and deconjugated bile salts were the only compound produced.
- Results from comparisons of amino acid sequences of known bacterial toxins with sequences of the predicted proteins from the genomic sequence of *B. longum* BB536 and genomic sequences of three known pathogens with sequences of the predicted proteins from the genomic sequence of *B. longum* BB536 indicate that there is no significant homology.
- *B. longum* BB536 was not observed to have hemolytic activity.

7) The LD_{50} of *B. longum* BB536 orally administered to mice was determined to be ~$5x10^{13}$ CFU/kg-bw. The LD_{50} of *B. longum* BB536 administered intraperitoneally to mice was determined to be ~$9x10^{11}$ CFU/kg-bw.

8) Results from repeat dose studies of *B. longum* BB536 administered to rats show no treatment effects on body weight, body weight gain, or feed intake at doses up to 2 x 10^{12} CFU/kg bw/day. Findings for the studies provide support for the safe use of *B. longum* BB536 under the test conditions.

9) Seventeen clinical studies (reported in 14 papers) involving the administration of *B. longum* BB536 to healthy adults were identified and reviewed. The duration of *B. longum* BB536 consumption ranged from 6 days to 14 weeks. In three studies, intakes of *B. longum* BB536 were approximately 10^{11} CFU per day; participants consumed this dose for periods of 4, 13 or 14 weeks. In all other human studies reviewed, doses were in the range of approximately 10^9 to 10^{10} CFU *B. longum* BB536 per day. None of the studies reported any participant dropouts or adverse events due to the test articles. Findings from the studies provide support for the safe and well-tolerated use of *B. longum* BB536 under the test conditions.

10) Eight clinical studies involving the administration of *B. longum* BB536 to unhealthy adults or children were identified and reviewed. Study durations ranged from 8 weeks to1 year. Daily viable *B. longum* BB536 intakes for adults were in the range of 10^9 to10^{10}

CFU in most studies, and approximately 10^{11} CFU B. *longum* BB536 per day in one study. Daily doses of *B. longum* BB536 in populations of children were approximately 10^9 CFU. None of the studies reviewed reported adverse events or patient dropouts as a result of *B. longum* BB536 supplementation. Findings from the studies provide support for the safe and well-tolerated use of *B. longum* BB536 under the test conditions.

11) Two studies involving the administration of *B. longum* BB536 to infants were identified and reviewed. In one study, preterm infants received $5x10^8$ CFU of *B. longum* via formula daily for 8 weeks. In another study term infants were administered $9x10^9$ CFU *B. longum* BB536/day over a 5-day period. Neither study reported adverse events due to *B. longum* BB536 supplementation. Findings from the studies provide support for the safe and well-tolerated use of *B. longum* BB536 under the test conditions.

12) Research on bifidobacteria has been conducted on several strains of *Bifidobacterium longum*; results from studies of other strains of *B. longum* provide corroborative evidence for the safety of human consumption of *B. longum* BB536.

13) While other bifidobacterial strains are not identical to *B. longum* BB536, they share many common characteristics. Therefore, studies on other bifidobacteria species commonly used as probiotics or in food production, such as *B. breve* and *B. infantis,* can be used to provide corroborative evidence for the safety of *B. longum* BB536. Results from acute studies in rats demonstrate that under conditions of the tests, neither *B. breve* nor *B. infantis* presented toxicological concerns at the highest doses tested. Results from subchronic toxicity studies of *B. infantis* and *B. breve* in rats demonstrate that under conditions of the tests, No Observed Adverse Effect Levels (NOAELs) were determined to be the doses tested. The doses tested were $2.3x10^{11}$ CFU *B. breve*/kg-bw/day and $7.6x10^{10}$ CFU *B. infantis*/kg-bw/day. Results from these studies of *B. breve* and *B. infantis* provide corroborative data to support the available evidence that *B. longum* BB536 is safe for human consumption.

14) Assuming addition of 1×10^{10} CFU of *B. longum* BB536 per serving of the target food categories, the estimated mean and 90th percentile 2-day average intakes of *B. longum* BB536 from all proposed use categories combined in the population ages 2 years and older are 7.5×10^{10} and 1.2×10^{11} CFU, respectively.

Determination of the GRAS status of *B. longum* BB536 under the intended conditions of use has been made through the deliberations of Joseph F. Borzelleca, Ph.D., D.A.B.T.; Mary Ellen Sanders, Ph.D.; Brooks Watt, M.D; Claire L. Kruger, Ph.D., D.A.B.T. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients.

These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of *B. longum* BB536 and the potential human exposure to *B. longum* BB536 resulting from its intended use as an ingredient in foods and have concluded:

> *There is no evidence in the available information on B. longum BB536 that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when B. longum BB536 is used at levels that might reasonably be expected from the proposed applications. B. longum BB536 is GRAS for use in foods as proposed by Morinaga Milk Industry Co, Ltd.*

Therefore, *B. longum* BB536 is safe and GRAS at the proposed levels of addition to foods. *B. longum* BB536 is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

E. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T.,CEO, Spherix Incorporated, 6430 Rockledge Drive, Westmoreland Bldg, Suite 503, Bethesda, Maryland, 20817, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ckruger@spherix.com.

F. SIGNATURE

Morinaga Milk Industry Co., Ltd. Hereby submits the notice of a GRAS Exemption Claim for *B. longum* BB536 under the intended contidions of use.

Signature
Claire L. Kruger, CEO, Spherix Inc.
Authorized Representative of Morinaga
Milk Industry Co., Ltd.

November 19, 2008
Date

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II. DESCRIPTION OF SUBSTANCE

A. COMMON OR USUAL NAME

Bifidobacterium longum BB536.

B. SYSTEMATIC IDENTIFICATION

The bacterium has been deposited with the American Type Culture Collection (ATCC) and is designated BAA-999^{TM} (ATCC 2007).

C. CLASSIFICATION

1. Morphology

Bifidobacterium longum BB536 (a strain of *B. longum)* is a Gram-positive and weakly positive anaerobic bacterium showing slightly curved pleomorphic bacilli. Cells are typically irregular or branched rods. Colonies are round, dark to light brown with smooth edges and surfaces raised in a convex or hemispherical shape.

2. DNA Sequence Similarity to Other Strains

DNA similarities between two strains of *B. longum* and of *B. animalis* were determined using a colorimetric microplate hybridization method. The similarity between *B. longum* and *B. animalis* genomic DNA was determined using the thermal melting point (T_m) method. The results of these two assays are reported in Table II-1. *B. longum* E194b^T was used to demonstrate the accurate identification of *B. longum* BB536 as a strain of the species *B. longum.* Because *B. animalis* is phenotypically similar to *B. longum,* it was necessary to demonstrate that the *Bifidobacterium longum* BB536 strain is similar to a *B. longum* reference strain at the genetic level and dissimilar to a reference strain of *B. animalis.* The results demonstrate that the *Bifidobacterium longum* BB536 strain is not a member of the species *B. animalis* and, while it is a member of the species *B. longum,* it is sufficiently dissimilar from the reference strain to warrant its own strain designation.

Table II-1. Genetic Characteristics of *Bifidobacterium longum* BB536		
% Similarity to BB536 Genomic DNA		**GC Content in *B. longum* BB536* (%)**
B. longum E194b[T1]	B. animalis R 101-8[T]	
82	13	62.2
* GC guanine-cytosine		

3. Phenotypic Identification

A carbohydrate fermentation pattern was determined. N-acetyl-β-glucosaminidase activity was measured colorimetrically by monitoring the release of nitrophenol from the substrate *p*-nitrophenyl-N-acetyl-β-glucosaminide. F6PPK activity was also measured. The results from these assays are presented in Table II-2.

[1] documentation for reference strain G. Reuter (1971)
http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=15707&Template=bacteria

Table II-2. Phenotypic Characteristics of *Bifidobacterium longum* BB536	
Test or Substance	**Result**
F6PPK activity	+
Gas from glucose	-
Arabinose	+
Xylose	+
Rhamnose	-
Ribose	-
Glucose	+
Mannose	+ S~ + S
Fructose	+
Galactose	+
Sucrose	+
Maltose	+
Cellobiose	-
Lactose	+
Trehalose	-
Melibiose	+
Raffinose	+S
Melezitose	+
Dextrin	±~+S
Starch	-
Glycogen	-
Inulin	-
Mannitol	-
Sorbitol	-
Inositol	-
Esculin	-
Salicin	-
a -Methylglucoside	±S
Sodium gluconate	-
N-acetyl-(3-glucosaminidase	+
Abbreviations: + (positive); —(negative); ± (very weakly positive); S (delayed reaction)	

D. PRODUCTION PROCESS

1. *Bifidobacterium longum* BB536 Original Culture

Bifidobacterium longum BB536, originally isolated from a healthy infant in 1969, was cultured in Briggs liver broth medium (pH 6.8) (Table II-3) and identified as a strain of *B. longum* by morphological observation, various physiological tests, and genetic analysis; the BB536

deposited in ATCC was the original culture isolated, and is the current strain subsequently used in the toxicology/clinical trials[2].

Table II-3. Composition of Briggs Liver Broth Medium

Substance	Amount in Medium
Tomato extract	400 ml
Neopeptone (Difco)	15 g
Yeast extract (Difco)	6 g
Liver extract	75 ml
Glucose	20 g
Soluble starch	0.5 g
NaCl	5 g
Tween 80	1 g
L-cysteine HCl H_2O	0.2 g
Pure water	525 ml

The original isolate of *B. longum* BB536 was resuspended in 10% reconstituted skim milk in glass vials. This culture was stored at -80°C in a deep freezer at Morinaga Laboratory as the original frozen culture of *B. longum* BB536. The maintenance of the original frozen culture has been tightly controlled in the Microbiology section of the Laboratory.

2. Culturing Process

a. Cultures A, B and C, Stock Cultures and Seed Cultures

Cells from the original frozen culture are thawed and inoculated into one of three media (A, B, or C) to create one of three cultures (A, B, or C). Each culture is then subcultured for several generations in order to condition the cells to each medium. After cells in cultures A, B and C are acclimated to their respective medium, a sample of each culture is added to a fresh sterile medium and stored in a deep freezer at Morinaga Laboratory as frozen stock cultures A, B and C, respectively. For production of commercial *Bifidobacterium longum* BB536 products in the Morinaga factory, frozen stock cultures are thawed and cultured as seed cultures. The three seed cultures of *B. longum* BB536 are inoculated into their respective medium and cultured for several generations for use as mother starter cultures. After culturing, medium pH, cell shape, and the absence of contaminants are determined for seed cultures A, B and C.

[2] http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=BAA-999&Template=bacteria

b. Mother Starter Culture

Activated seed cultures are inoculated into the appropriate medium and cultured to make the first mother starters, which are then used in scale-up procedures designed to create large cultures for production. Each sterilized fresh medium is inoculated with the same main starter to prepare the second mother starter. Medium is inoculated with each second cultured mother starter and cultured to get the third mother starter. The respective culture media are identical to the seed culture media described above. After each step in the scale-up process, each mother culture is cooled and stored until used to prepare the next culture. After each step in the scale-up process, medium pH, density (Cultures A and B) and cell shape are analyzed for quality control.

c. Main Starter Cultures and Manufacturing Cultures

Sterilized fresh medium is inoculated with each mother starter culture in an industrial fermentation jar and cultured to prepare each main starter culture. Each main starter medium for cultures A, B and C is identical to the mother and seed culture media. After cultivation, the culture solutions are cooled and kept at a constant temperature until used in the manufacturing process. When ready for manufacturing, fresh medium is inoculated with the main starter, cultured and subsequently cooled and evaluated for *B. longum* BB536 cell counts, cell shape, medium pH and bioburden.

3. Non-Culturing Process

Depending on which culture and medium is used, each manufacturing culture goes through a series of steps including large-scale concentration, washing, re-concentration, mixing, rapid freezing or freeze drying and powdering. Powdered *Bifidobacterium longum* BB536 is then mixed with one of three carriers (1, 2 or 3) depending on what final product it will be made into. Sifting, weighing and packaging, metal checking and storage complete the production process.

4. Quality Control

Every lot *of Bifidobacterium longum* BB536 product is tested for quality control purposes. Descriptions of the tests performed and the specifications for each product are discussed in the next section.

E. FINISHED PRODUCT DESCRIPTIONS

Finished products made with *B. longum* BB536 cultures include FC-B, FCB-M1, BIFILON-50F,

BIFILON-50N, BIFILON-50T, BIFILON-EX, and BIFILON-EX/LM. These finished products have different properties and are used for different types of products.

- FC-B is used in the production of Bifidus milk products and yogurt.
- FCB-M1 is used as a yogurt starter.
- BIFILON-50F is the only BIFILON manufactured without milk-based ingredients. The other BIFILON powders are produced with milk protein.
- BIFILON-50N is used in health foods. It contains cornstarch as a carrier.
- BIFILON-50T contains tapioca starch as a carrier.
- BIFILON-EX contains potato starch as a carrier. It contains high levels of milk proteins.
- BIFILON-EX/LM was developed for customers who require low milk content and high *B. longum* BB536 cell counts.

The specifications for BIFILON-50F, 50N, and 50T require more than 80 billion cells of *B. longum* BB536 per gram of finished product. The specification for BIFILON-EX and BIFILON-EX/LM requires more than 150 billion cells *B. longum* BB536 per gram of finished product. The carriers mixed with the *B. longum* BB536 finished product are all food-grade.

F. PRODUCT CHARACTERIZATION

1. Verification of *Bifidobacterium longum* BB536 Cells in Seed Culture Media

Morinaga undertook a study to demonstrate that the *B. longum* BB536 cells used to produce seed cultures for Morinaga products are identical to the original *B. longum* BB536 cells isolated in 1969. The electrophoretic patterns of RAPD-PCR products of all seed cultures were identical to the original *Bifidobacterium longum* BB536 culture. These results demonstrate that the *B. longum* BB536 cells used in each seed culture are identical to the original *B. longum* BB536 culture. Because the cells are the same, safety studies conducted with the original *B. longum* BB536 culture are relevant to the safety of the *B. longum* BB536 cells found in the products at issue.

2. Specifications and Lot Data for Finished Products of *Bifidobacterium longum* BB536

The tests and specifications for the final products made from *Bifidobacterium longum* BB536

cultures are described in Tables II-4 to II-10. Five lots of the seven different products containing *Bifidobacterium longum* BB536 were analyzed using multiple analytical procedures.

Table II-4. FC-B Specifications and Lot Data

		Lot No. (Indicates End of Shelf Life Date)					
		040123	050612	060123	060210	060704	
		Production Date*					
Parameter	**Standard Specification**	**2003.10.23**	**2005.03.12**	**2005.10.23**	**2005.11.10**	**2006.04.04**	**Analytical Method**
Appearance	Normal	Passed	Passed	Passed	Passed	Passed	Visual check
Foreign bodies	Negative	Passed	Passed	Passed	Passed	Passed	Filtration
Bifidobacterium cell counts	> 8.0 x10^{10}/g	1.0 x 10^{11}/g	1.1 x 10^{11}/g	9.8 x 10^{10}/g	9.6 x 10^{10}/g	1.1 x 10^{11}/g	MGL medium, BL agar medium or RCM
Total aerobes	< 10 cfu/g	Passed	Passed	Passed	Passed	Passed	SPC medium
Coliform	Negative	Passed	Passed	Passed	Passed	Passed	DESO or BGLB medium
Psychrotrophic bacteria	< 10 per g	Passed	Passed	Passed	Passed	Passed	SPC medium, 15°C, 5 days incubation
Mold	30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
Yeast	30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium

Abbreviations:
BGLB Brilliant Green Lactose Bile Broth
BL Blood liver
DESO Desoxycholate agar
MGL Modified Garche's agar with Lithium Chloride
RCM Reinforced Clostridial Medium
SPC Standard Plate Count

Notes:
Source: Information obtained via personal communication with Mr. Abe, Morinaga Milk Industry Co., Ltd. (May 2007).
* Calculated based on 3-month shelf life for FC-B as indicated by Mr. Abe, Morinaga Milk Industry Co., Ltd. (May 2007).

Table II-5. FCB-M1 Specifications and Lot Data

		Lot No. (Indicates End of Shelf Life Date)					
		031108	040203	041010	060805	070808	
		Production Date*					
Parameter	**Standard Specification**	**2002.11.08**	**2003.02.03**	**2003.10.10**	**2005.08.05**	**2006.08.08**	**Analytical Method**
Appearance	Normal	Passed	Passed	Passed	Passed	Passed	Visual check
Foreign bodies	Negative	Passed	Passed	Passed	Passed	Passed	Filtration
Bifidobacterium cell counts	> $3.5x10^{10}$/g	4.0×10^{10}/g	3.8×10^{10}/g	4.3×10^{10}/g	3.9×10^{10}/g	4.2×10^{10}/g	MGL medium, BL agar medium or RCM
Total aerobes	< 10 cfu/g	Passed	Passed	Passed	Passed	Passed	SPC medium
Coliform	Negative	Passed	Passed	Passed	Passed	Passed	DESO or BGLB medium
Psychrotrophic bacteria	< 10 per g	Passed	Passed	Passed	Passed	Passed	SPC medium, 15°C, 5 days incubation
Mold	< 30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
Yeast	< 30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium

Abbreviations:
BGLB Brilliant Green Lactose Bile Broth
BL Blood liver
DESO Desoxycholate agar
MGL Modified Garche's agar with Lithium Chloride
RCM Reinforced Clostridial Medium
SPC Standard Plate Count

Notes:
Source: Information obtained via personal communication with Mr. Abe, Morinaga Milk Industry Co., Ltd. (May 2007).
* Calculated based on 3-month shelf life for FC-B as indicated by Mr. Abe, Morinaga Milk Industry Co., Ltd. (May 2007).

Table II-6. BIFILON-50F Specifications and Lot Data

Parameter	Standard Specification	Lot No. (Indicates Production Date)					Analytical Method
		2006.05.26	2006.06.05	2006.06	2006.07.14	2006.08.01	
B. longum BB536 Identity	Identified	Passed	Passed	Passed	Passed	Passed	RAPD method
Appearance	Normal	Passed	Passed	Passed	Passed	Passed	Visual check
Foreign bodies	Negative	Passed	Passed	Passed	Passed	Passed	Visual check
Moisture	< 6 %	Passed	Passed	Passed	Passed	Passed	105 °C, 4 hr
Bifidobacterium cell counts	> 8.0×10^{10}/g	1.2×10^{11}/g	1.1×10^{11}/g	1.0×10^{11}/g	1.0×10^{11}/g	1.2×10^{11}/g	BL agar medium or RCM
Total aerobes	<500 cfu/g	Passed	Passed	Passed	Passed	Passed	SPC method
Coliform	Negative per 10g x 2 times	Passed	Passed	Passed	Passed	Passed	Enrichment medium and VRBD
Mold	< 50 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
Yeast	< 50 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
S. aureus	< 100 per 1 g x 5 times	Passed	Passed	Passed	Passed	Passed	Vogel Johnson agar
Salmonella	Negative per 25 g x 8	Passed	Passed	Passed	Passed	Passed	Selenite Enrichment broth
Milk protein(βLG)	< 10 ppm	Passed	Passed	Passed	Passed	Passed	Sandwich ELISA
Arsenic	< 1 ppm	Passed	Passed	Passed	Passed	Passed	Gutzeit method
Heavy metal	< 5 ppm	Passed (<2 ppm)	Passed (<2 ppm)	Passed (<2 ppm)	Passed (<2 ppm)	Passed (<2 ppm)	Colorimetric method
Lead	<0.5 ppm	Passed	Passed	Passed	Passed	Passed	Atomic absorption spectroscopy

000022

Abbreviations:

BL	Blood Liver
RAPD	Rapidly amplified polymorphic DNA
RCM	Reinforced Clostridial Medium
SPC	Standard Plate Count
VRBD	Violet Red Bile agar with Dextrose

Notes:

Source: Information obtained via personal communication from Mr. Abe, Morinaga Milk Industry Co., Ltd. (May 2007)

Table II-7. BIFILON-50N Specifications and Lot Data

Parameter	Standard Specification	Lot No. (Indicates Production Date)					Analytical Method
		2006.03.03	2006.03.28	2006.06.19	2006.07.11	2006.07.25	
Appearance	Normal	Passed	Passed	Passed	Passed	Passed	Visual check
Foreign bodies	Negative	Passed	Passed	Passed	Passed	Passed	Visual check
Moisture	< 6%	Passed	Passed	Passed	Passed	Passed	105° C, 4 hr
Bifidobacterium cell counts	> 8.0×10^{10}/g	1.1×10^{11}/g	1.2×10^{11}/g	1.1×10^{11}/g	1.3×10^{11}/g	1.1×10^{11}/g	BL agar medium or RCM
Total aerobes	< 300 cfu/g	Passed	Passed	Passed	Passed	Passed	SPC medium
Coliform	Negative per 0.2 g	Passed	Passed	Passed	Passed	Passed	DESO or BGLB medium
Mold	< 30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
Yeast	< 30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
S. aureus	< 100 per g	Passed	Passed	Passed	Passed	Passed	Vogel Johnson agar
Arsenic	< 1 ppm	Passed	Passed	Passed	Passed	Passed	Gutzeit method
Heavy metal	< 5 ppm	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Colorimetric method
Lead	<0.5 ppm	Passed	Passed	Passed	Passed	Passed	Atomic absorption spectroscopy

Abbreviations:
BGLB Brilliant Green Lactose Bile Broth
BL Blood liver
DESO Desoxycholate agar
RCM Reinforced Clostridial Medium
SPC Standard Plate Count
Notes:
Source: Information obtained via personal communication with Mr. Abe, Morinaga Milk Industry Co., Ltd. (May 2007).

000024

Table II-8. BIFILON-50T Specifications and Lot Data

Parameter	Standard Specification	Lot No. (Indicates Production Date)					Analytical Method
		2005.05.24	2005.09.13	2005.11.29	2006.06.19	2006.07.25	
Appearance	Normal	Passed	Passed	Passed	Passed	Passed	Visual check
Foreign bodies	Negative	Passed	Passed	Passed	Passed	Passed	Visual check
Moisture	< 6%	Passed	Passed	Passed	Passed	Passed	105° C, 4 hr
Bifidobacterium cell counts	> 8.0×10^{10}/g	1.3×10^{11}/g	1.2×10^{11}/g	1.1×10^{11}/g	9.7×10^{10}/g	1.3×10^{11}/g	BL agar medium or RCM
Total aerobes	< 300 cfu/g	Passed	Passed	Passed	Passed	Passed	SPC medium
Coliform	Negative per 0.2 g	Passed	Passed	Passed	Passed	Passed	DESO or BGLB medium
Mold	< 30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
Yeast	< 30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
S. aureus	< 100 per g	Passed	Passed	Passed	Passed	Passed	Vogel Johnson agar
Arsenic	< 1 ppm	Passed	Passed	Passed	Passed	Passed	Gutzeit method
Heavy metal	< 5 ppm	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Colorimetric method
Lead	<0.5 ppm	Passed	Passed	Passed	Passed	Passed	Atomic absorption spectroscopy

Abbreviations:
BGLB Brilliant Green Lactose Bile Broth
BL Blood liver
DESO Desoxycholate agar
RCM Reinforced Clostridial Medium
SPC Standard Plate Count
Notes:
Source: Information obtained via personal communication with Mr. Abe, Morinaga Milk Industry Co., Ltd. (May 2007).

Table II-9. BIFILON-EX Specifications and Lot Data

Parameter	Standard Specification	Lot No. (Indicates Production Date)					Analytical Method
		2005.03.24	2005.07.15	2005.09.06	2005.12.13	2006.04.11	
Appearance	Normal	Passed	Passed	Passed	Passed	Passed	Visual check
Foreign bodies	Negative	Passed	Passed	Passed	Passed	Passed	Visual check
Moisture	< 6%	Passed	Passed	Passed	Passed	Passed	105° C, 4 hr
Bifidobacterium cell counts	> $1.5x10^{11}$/g	2.7×10^{11}/g	2.2×10^{11}/g	2.3×10^{11}/g	2.5×10^{11}/g	2.1×10^{11}/g	BL agar medium or RCM
Total aerobes	< 300 cfu/g	Passed	Passed	Passed	Passed	Passed	SPC medium
Coliform	Negative per 0.2 g	Passed	Passed	Passed	Passed	Passed	DESO or BGLB medium
Mold	< 30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
Yeast	< 30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
S. aureus	< 100 per g	Passed	Passed	Passed	Passed	Passed	Vogel Johnson agar
Salmonella	Negative per 5 g	Passed	Passed	Passed	Passed	Passed	Selenite enrichment broth
Listeria	Negative per 25 g	Passed	Passed	Passed	Passed	Passed	Listeria enrichment broth
Arsenic	< 1 ppm	Passed	Passed	Passed	Passed	Passed	Gutzeit method
Heavy metal	< 5 ppm	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Colorimetric method
Lead	<0.5 ppm	Passed	Passed	Passed	Passed	Passed	Atomic absorption spectroscopy

Abbreviations:
BGLB Brilliant Green Lactose Bile Broth
BL Blood liver
DESO Desoxycholate agar
RCM Reinforced Clostridial Medium
SPC Standard Plate Count
Notes:
Source: Information obtained via personal communication with Mr. Abe, Morinaga Milk Industry Co., Ltd. (May 2007).

Table II-10. BIFILON-EX/LM Specifications and Lot Data

Parameter	Standard Specification	Lot No. (Indicates Production Date)					Analytical Method
		2004.07.06	2005.01.25	2005.05.24	2005.08.02	2006.03.28	
Appearance	Normal	Passed	Passed	Passed	Passed	Passed	Visual check
Foreign bodies	Negative	Passed	Passed	Passed	Passed	Passed	Visual check
Moisture	< 6%	Passed	Passed	Passed	Passed	Passed	105° C, 4hr
Bifidobacterium cell counts	> $1.5x10^{11}$/g	1.8×10^{11}/g	2.3×10^{11}/g	2.3×10^{11}/g	2.1×10^{11}/g	1.8×10^{11}/g	BL agar medium or RCM
Total aerobes	< 300 cfu/g	Passed	Passed	Passed	Passed	Passed	SPC medium
Coliform	Negative per 0.2 g	Passed	Passed	Passed	Passed	Passed	DESO or BGLB medium
Mold	< 30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
Yeast	< 30 per g	Passed	Passed	Passed	Passed	Passed	Potato dextrose medium
S. aureus	< 100 per g	Passed	Passed	Passed	Passed	Passed	Vogel Johnson agar
Salmonella	Negative per 5 g	Passed	Passed	Passed	Passed	Passed	Selenite enrichment broth
Listeria	Negative per 25 g	Passed	Passed	Passed	Passed	Passed	Listeria enrichment broth
Arsenic	< 1 ppm	Passed	Passed	Passed	Passed	Passed	Gutzeit method
Heavy metal	< 5 ppm	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Passed (< 2 ppm)	Colorimetric method
Lead	<0.5 ppm	Passed	Passed	Passed	Passed	Passed	Atomic absorption spectroscopy

Abbreviations:
BGLB Brilliant Green Lactose Bile Broth
BL Blood liver
DESO Desoxycholate agar
RCM Reinforced Clostridial Medium
SPC Standard Plate Count
Notes:
Source: Information obtained via personal communication with Mr. Abe, Morinaga Milk Industry Co., Ltd. (May 2007).

3. Analytical Methods

a. Random Amplified Polymorphic DNA (RAPD) Method

Bifidobacterium longum BB536 products (e.g., BIFILON-50F) are analyzed using a RAPD method to ensure that *B. longum* BB536 is present. The procedure subsequently described was provided by Morinaga Milk Industry Co., Ltd via email correspondence in May 2007.

1. The product (e.g., BIFILON-50F) is suspended in pure water and gentle centrifugation is used to remove the starch present in the sample. *Bifidobacterium longum* BB536 cells are then harvested by centrifugation and resuspended in pure water. This washing process is repeated twice. After washing, the cell pellet is suspended in lysis solution [20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2% Triton X-100] including Lysozyme (20 mg/ml) to facilitate cell lysis.

2. DNA extraction and purification are performed using a DNeasy Tissue Kit (QIAGEN, Germany)

3. A PCR primer with the sequence AACGCGC AAC is used.

4. 25 µL of amplification reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3 mM MgCl2, 250 µM dNTPs (each), 30 pmol of primer, 2U of Taq DNA polymerase, and approximately 100 ng of sample DNA are prepared.

5. The thermocycle program consists of the following:
   a) 4 cycles of 5 min at 94°C, 5 min at 36°C, and 5 min at 72°C;
   b) 30 cycles of 1 min at 94°C, 1 min at 36°C, and 1 min at 72°C;
   c) 72°C for 10 min.

6. PCR products are electrophoresed in 1.8 % (wt/vol) agarose gel-TBE buffer, stained with ethidium bromide, and photographed. A 100-bp DNA ladder is used as a size reference.

7. The electrophoretic pattern of amplified DNA is compared to a reference standard.

b. Enumeration Method

The enumeration method is used to determine the number of viable cells in cultures of *Bifidobacterium longum* BB536. One gram of product is thoroughly dissolved in a sterilized buffer solution containing the substances listed in the Table II-11.

Table II-11. Buffer Solution Used for Enumeration Method

Substance	Amount in Solution (g)
KH_2PO_4	4.5
Na_2HPO_4	6.0
L-Cysteine.HCl.H_2O	0.5
Tween 80	0.5
Agar	1.0
Water (mL)	1000

The buffer solution is heated to approximately 35°C before use. The sample is then sequentially diluted with either the same buffer solution or saline. The diluted solution (0.1-0.2 ml), which should contain 30-300 viable cells, is spread on a BL agar plate or mixed with BL agar medium. Alternatively, reinforced clostridial agar medium (Oxoid, UK) may be used. The plate is incubated anaerobically for 72 hr at 37°C. One of three anaerobic conditions is used: (1)100% carbon dioxide gas, (2) a gas mixture of 80% nitrogen, 10% hydrogen and 10% carbon dioxide or (3) a commercially available anaerobic culturing kit. After incubation, the colonies are counted and the number of viable cells in the sample is calculated.

4. Stability *of Bifidobacterium longum* BB536 in BIFILON Finished Products

Morinaga evaluated the survival of *Bifidobacterium longum* BB536 in the BIFILON products: BIFILON-50N, BIFILON-EX and BIFILON 50F over 18 to 36-month periods. The BIFILON products serve as intermediates that are subsequently used to make market products. Stability studies demonstrate acceptable stability.

5. Stability of *Bifidobacterium longum* BB536 in Various Market Products

Morinaga also evaluated the survival *of Bifidobacterium longum* BB536 in market products to identify survival of the organism over shelf life of the products. Stability studies on products including chocolate, gum, ready-to-eat breakfast cereals, nutrition powder (for Tofu) and protein powder were conducted for 18 to 24-month periods. Milk and yogurt were studied for 21 days. The percent survival of *B. longum* BB536 for each product at the end of shelf life was determined. The percent survival data were then used to calculate overages of *B. longum* BB536

at the time of market product production necessary to ensure target levels of the organism throughout shelf life. This information is presented in Table II-12. The calculated range of overages, as a multiple of the target *B. longum* BB536 concentration, was determined to be approximately 1 to 10.

Table II-12. Estimated *B. longum* BB536 Overages in Market Products

Food	Subcategories	Survival Rate (%)[a] Beginning of Shelf Life	Survival Rate (%)[a] End of Shelf Life	Overage[b]
Chocolate	block	100	80	1.3
Chocolate	flake	100	80	1.3
Gum	NA[c]	100	25	4.0
RTE cereal	cocoa rice krispies	100	50	2.0
RTE cereal	fruit oat bran flakes	100	40	2.5
Powder (tofu)	vanilla	100	40	2.5
Powder (tofu)	chocolate	100	30	3.3
Protein Powder	NA	100	60	1.7
Milk	whole	100	55	1.8
Milk	low fat	100	45	2.2
Yogurt	regular yogurt	100	10[d]	10.0
Yogurt	drink yogurt	100	31.6[d]	3.1
		Range of overage	Min	1.3
			Max	10

[a] Values taken from graphs in DRAFT GRAS Determination for BB536; Figure 3, pages 38-42 (May 16, 2007).
[b] Overages are presented as multiples of the intended use level that must be added at beginning of shelf life to ensure that *B. longum* BB536 levels at end of shelf life are equivalent to intended use levels.
[c] NA = not applicable.
[d] Values derived from log-scale graph.

6. Analysis of Market Product

Morinaga Caldus is a milk product that contains lactobacilli and *B. longum* BB536. It is marketed in Japan and has FOSHU status. To analyze the consistency in the composition of Morinaga Caldus, a 10^{-1} dilution was prepared. Using this as the stock, serial 10-fold dilutions were prepared. Depending on the viable count of the sample, 2 to 3 dilutions were made and 0.1

ml of each dilution was spread evenly on an agar plate and incubated. After confirming that the colonies were *B. longum* BB536, colonies were counted. The tests of general composition and determination of lactobacilli and bifidobacteria were conducted according to "Analytical Methods for Nutritional Compositions etc. in Nutrition Labeling Standards" (Notification from head of Newly-developed Food Sanitation Measures Office, Department of Food Sanitation, Environmental Health Bureau, Ministry of Health Labour and Welfare, Eishin No. 13, April 26, 1999, as cited by Morinaga Milk Industry Co., Ltd.). Table II-13 presents the results of the composition analysis of the Morinaga Caldus.

Table II-13. Composition of Morinaga Caldus Market Product

Parameter & Unit	Sample Lot No (& Sampling Date)			Test Method
	6 (Jun-29-00)	7 (Jun-30-00)	8 (Jul-02-00)	
B. longum BB536 (cfu/g)	2.6×10^7	2.6×10^7	2.6×10^7	BL agar method
Calories (Kcal/100g)	59	59	59	Calculated[(a)]
Water content (g/100g)	86.5	86.5	86.5	Normal pressure heat drying method
Protein (g/100g)	4.7	4.7	4.7	Kjeldahl method (nitrogen protein conversion coefficient: 6.25)
Fats (g/100g)	2.0	2.0	2.1	Röse-Gottlieb method
Carbohydrate (g/100g)	5.6	5.6	5.5	Calculated[(b)]
Dietary Fiber (g/100g)	0.2	0.2	0.2	Enzymatic/weight method
Ash content (g/100g)	1.0	1.0	1.0	Direct ashing method
Sodium (mg/100g)	69	70	69	Atomic absorption spectroscopy
Calcium (mg/100g)	190	210	190	Atomic absorption spectroscopy
Iron (mg/100g)	0.6	0.6	0.6	Ortho-phenanthroline absorptiometric method
Lactobacillus and bifidobacteria cell counts (CFU/mL)	2.7×10^7	3.0×10^7	2.7×10^7	Method provided by consignor (Method according to ministerial ordinance concerning standards of milk and diary products)

Abbreviations:
BL Blood liver
Notes:
Source: Morinaga Milk Industry Co., Ltd.
(a) Calorie (kcal/100 g) = protein x 4 + fats x 9 + carbohydrate x 4
(b) Carbohydrate = 100 - water content - protein - fats - dietary fiber - ash content

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7. Stability of *Bifidobacterium longum* BB536 in Dairy Products

Morinaga performed studies to determine the stability of various bifidobacteria in bifidobacteria-containing yogurt and milk products. Schematics of the procedures conducted in yogurt and milk products are presented in Figures 1 and 2, respectively.

Figure 1. Processing of Yogurt Products Containing Bifidobacteria

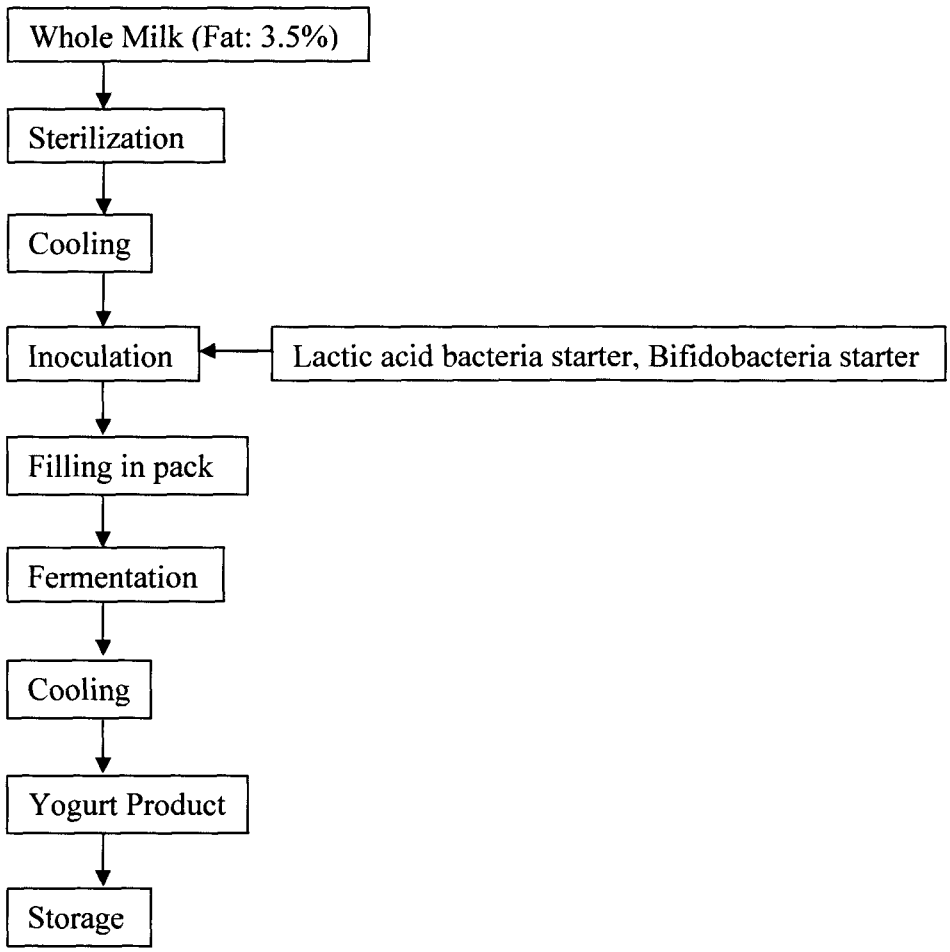
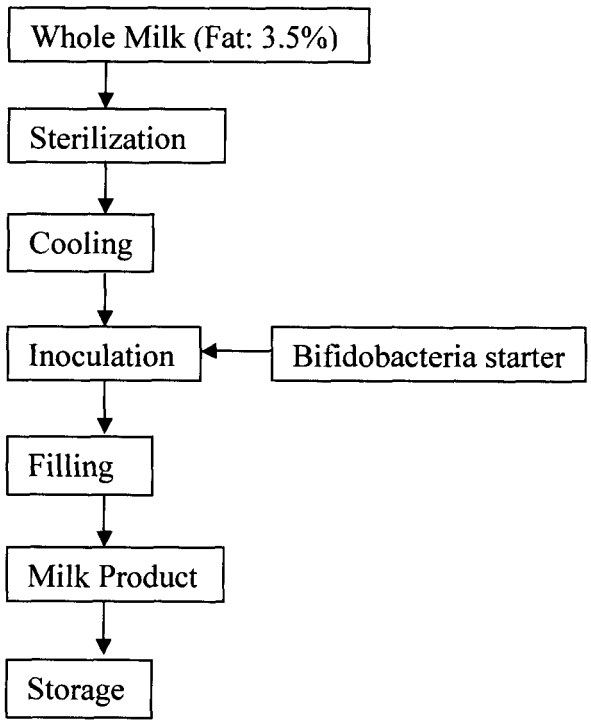


Figure 2. Processing of Milk Products Containing Bifidobacteria



a. Results

The numbers of lactobacilli in the yogurts ranged from 5 to 10 x 10^8 per ml and did not change significantly during the storage period. In all yogurts prepared with bifidobacteria, the bifidobacteria concentration reached 10^8 per ml or higher one day after production. Significant differences in concentration were observed among the bifidobacterial strains after prolonged storage. The yogurt containing *B. longum* BB536 maintained a viable cell concentration of over 10^8 per ml after 14 days of storage.

In bifidobacteria-containing milks, milk containing *B. longum* BB536 had 79% of the original concentration measured on the day of manufacture after 14 days of storage.

b. Conclusion

In bifidobacteria-containing food products, the concentration of bifidobacteria is an important factor that can vary throughout the shelf life of the product. Among the bifidobacteria-containing yogurts tested, the *B. longum* BB536 strain had a high rate of survival after 14 days of storage, which is the usual expiration date for conventional yogurts.

8. Stability of "Morinaga Bifidus"

Morinaga conducted storage tests on a dairy foods product containing *B. longum* BB536 and *Lactobacillus* with the commercial name "Morinaga Bifidus." Samples from three different manufacturing days were selected for evaluation of product content. Storage conditions consisted of refrigeration of the product at 10°C for 10 days. Analyses indicate that the levels *of Bifidobacterium longum* BB536 at day 10 were approximately the same as at day 1.

9. Stability of Morinaga Caldus

Morinaga also conducted storage tests on "Morinaga Caldus." Samples from three different manufacturing days were selected for analysis. Storage conditions consisted of refrigeration of the product at 10°C for 10 days. Analyses indicate that levels of *Bifidobacterium longum* BB536 at day 10 exceeded or were approximately the same as at day 1.

III. HISTORY OF USE

A. HISTORICAL EXPOSURE TO BIFIDOBACTERIA

1. Naturally Occurring in Humans

Bifidobacteria are a natural component of the normal human gut miroflora. Bifidobacteria comprise up to 25% of the cultivatable fecal bacteria in adults and 80% in infants (Picard et al. 2005), and *Bifidobacterium longum* has been detected in feces from infants and adults (Benno et al. 1984, Benno et al. 1989). *Bifidobacterium longum* BB536, the subject of this GRAS determination, was isolated from a healthy infant.

2. Bifidobacteria Added to Foods

Lactic acid bacteria, including lactobacilli and bifidobacteria, are commonly consumed in fermented foods throughout the world. In the United States, yogurts are required to contain *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (21 CFR §131.200, 203, 206) and some yogurts also contain *Bifidobacterium* species (Sanders 2003). Prior sanctions were granted for the use of harmless lactic acid producing bacteria as optional ingredients in specified standardized foods including cultured milk, sour cream, cottage cheese, and yogurt (FDA 2001). Breads, rolls and buns also may contain lactic-acid producing bacteria as optional ingredients (21 CFR §136.110).

Bifidobacterium longum BB536 was first commercially available in Japan in 1977 with the launch of Morinaga Bifidus Milk. At present, several products containing *B. longum* BB536 are available on the Japanese market. The availability of *B. longum* BB536 in Europe began in 1986 with the production of Morinaga Bifidus Yogurt in France, followed by Sweden in 1989. In 1994, *B. longum* BB536 was sold as a frozen starter to European dairy companies in Germany, Russia, Poland and other countries within the EU. The commercial history of *B. longum* BB536 is summarized in Table III-1.

Table III-1. Commercial History of BB536	
Date	**Product**
1977	Morinaga Bifidus Milk (Japan)
1979	Morinaga Bifidus Yogurt (Japan)
1991	Morinaga Caldus (calcium-enriched BB536 milk)
1994	BB536 starter culture (BB536 fermented milk use in Europe)
1999	BB536 supplement use by marketers in the U.S.A.
2005	BB536 Capsule (Japan)
2006	BB536 "Growing-up Milk" follow-on formula (Indonesia)
Source: Morinaga Milk Industry Co., Ltd.	

Several other strains of *Bifidobacterium* are commercially available for probiotic uses. Examples of commercially available strains are shown in Table III-2.

Table III-2. Commercially Available Probiotic *Bifidobacterium* Strains	
Strain	**Source**
B. animalis DN-114 001	Dannon
B. breve strain Yakult*	Yakult
B. infantis 35624	Procter & Gamble
B. lactis Bb-12	Chr. Hansen, Inc.
B. lactis DR10 (HN019)	Fonterra Cooperative Group Ltd.
B. longum BB536*	Morinaga Milk Industry Co., Ltd. (Zama-City, Japan)
B. longum SBT-2928*	Snow Brand Milk Products Co., Ltd.
Source: Sanders 2003, Dannon (2007), Nestle (2007), Procter & Gamble (2007). * Strains approved for functional food use by the Ministry of Health in Japan.	

Dannon® currently markets two products in the U.S. and other countries that contain supplemental lactic acid bacteria. Yogurts in the "Activia" product line provide over 10^{10} live cultures including *Bifidobacterium animalis* DN-173 010, *L. bulgaricus* and *S. thermophilus* per 4 ounce serving (Dannon 2007a). "DanActive", a dairy drink also produced by Dannon®, contains 10^{10} live*Lactobacillus casei* DN-114 001 cultures per 3.3 fluid ounce serving; the product also contains *L. bulgaricus* and *S. thermophillus* (Dannon 2007b).

The addition of *Bifidobacterium lactis* strain Bb12 to formulas for infants four months of age and older has been determined to be Generally Recognized As Safe (GRAS). In 2002, the U.S. Food and Drug Administration indicated that they had no questions about the GRAS notification for this use as submitted by Nestlé® (FDA 2002). The intended use of freeze-dried *B. lactis* strain Bb12 in formula for infants four months and older is 10^7 - 10^8 CFU/g in the final powdered product, and the intended level of resuscitated organisms is within the range of 10^6 - 10^8 CFU/g of finished formula.

In 2007, Nestle® introduced the first infant formula for consumption by infants aged 0 to 4 months (Nestle 2007) containing bifidobacteria to the U.S. market. The product, "Good Start Natural Cultures" contains 10^6 CFU *Bifidobacterium lactis* Bb12 per g of powder.

3. Dietary Supplements

According to the Council for Responsible Nutrition (and based on a list compiled by the National Nutritional Foods Association), *Bifidobacterium longum, Bifidobacterium bifidum* and *Bifidobacterium infantis* have been "grandfathered in" as dietary supplement ingredients under the stipulations set forth by the Dietary Supplement and Health and Education Act (DSHEA) of 1994 (CRN 1998). *B. longum* BB536 has been commercially available in the U.S. as a dietary supplement since 1991. The capsules are available in two sizes. One contains 3 x 10^9 CFU *B. longum* BB536; the other provides more than 1 x 10^{10} CFU *B. longum* BB536 per capsule. The recommended use for both capsule sizes is one capsule up to three times per day, resulting in a maximum intake of approximately 9 x 10^9 CFU or 3 x 10^{10} CFU *B. longum* BB536 per day.

A New Dietary Ingredient (NDI) notification was filed with the U.S. Food and Drug Administration for *Bifidobacterium infantis* strain 35624 by The Procter & Gamble Company (FDA 2005). The recommended intake of the supplement is up to 10^{10} CFU/d for healthy adults. Procter & Gamble currently markets this probiotic under the brand name "Align." Each capsule provides 10^9 CFU *B. infantis* over the course of recommended shelf-life (Procter and Gamble 2007).

B. Intended Uses and Estimated Intakes of *B. longum* BB536

1. Intended Uses

Morinaga intends to add *B. longum* BB536 to a wide variety of selected foods in the U.S. food supply. The specific product categories to which *B. longum* BB536 will be added are identified in Table III-3. The intended use in all product categories is 1 x 10^{10} CFU *B. longum* BB536 per serving of product. Typical serving sizes for the intended uses correspond to the Reference Amounts Customarily Consumed (RACC) (21 CFR 101.12). *B. longum* BB536 will be added to finished foods to ensure that the products contain the intended levels of viable bacteria. For example, *B. longum* BB536 is sprayed on breads and baked products after baking and is therefore not subjected to the high heat required to make these products.

2. Estimated Intakes of *B. longum* BB536

Estimates of potential intake of *B. longum* BB536 resulting from the intended uses of the organism in select foods were calculated using food consumption data reported in the United States Department of Health and Human Service's 2003-2004 National Health and Nutrition Examination Survey (NHANES). The NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the United States (NCHS 2006).

As part of the examination, trained dietary interviewers collect detailed information on all foods and beverages consumed by respondents in the previous 24 hour time period (midnight to midnight). A second dietary recall was administered by telephone 3 to 10 days after the first dietary interview, but not on the same day of the week as the first interview. A total of 9043 respondents provided complete dietary intakes for the Day 1 recall, and 8354 of the individuals provided a complete Day 2 recall.

The data files used to process the NHANES 2003-2004 dietary recalls include 6940 food codes, each identified by a unique number and descriptive name. The food codes represent single component foods or ingredients such as milk or vegetable oil; finished foods such as bread or margarine; and also food mixtures such as a grilled cheese sandwich. The database was reviewed, and all food codes corresponding to one of the intended use categories of *B. longum* BB536 were identified. USDA survey files (USDA 2006) were used to identify the proportion of food mixtures represented by applicable use categories. For example, food codes for "cheeseburger" may be included in estimates of the bread/roll, processed cheese, and catsup use categories based on the percentage (by weight) of each component in the USDA files corresponding to the "cheeseburger" food codes.

Using the list of food codes and the NHANES 2003-2004 dietary recall data files from individuals with 2 complete days of dietary recall, estimates of the mean and 90th percentile 2-day average intakes of the individual product categories and also all categories combined was completed. Two-day average intakes represent the total number of servings consumed during the two days of recall divided by two (i.e., ($Intake_{Day\ 1}$ + $Intake_{Day\ 2}$)/2).

Intakes were calculated for subpopulations of infants (< 1 y M+F, 1 y M+F), children (2-5 y M+F, 6-11 y M and F separately), teenagers (12-18 y M and F separately) and adults (19+ y M and F separately). The estimates were generated using survey sample weights to adjust for differences in representation of subpopulations; results therefore are representative of the U.S. population.

Estimates of intakes in terms of average number of food servings per day are shown in Table III-4. The population ages 2 years and older consumed a mean of 7.5 servings of the target foods per day, while the 90th percentile of intake was 12.5 servings per day. Among the subpopulations, mean 2-day average intakes from all categories combined ranged from 2.4 to 9.3 servings per day, and 2-day average 90th percentile intakes ranged from 5.3 to 15.3 servings per day.

Assuming addition of 1 x 10^{10} CFU of *B. longum* BB536 per serving of the target food categories, i.e., the target level of *B. longum* BB536 at the end of market product shelf life, the estimated mean and 90th percentile 2-day average intakes of *B. longum* BB536 from all categories combined in the population ages 2 years and older are 7.5 x 10^{10} and 1.2 x 10^{11} CFU, respectively. Across all of the subpopulations in this analysis, the maximum estimated 2-day average 90th percentile intake of *B. longum* BB536 from all categories combined is approximately 1.5 x 10^{11} CFU; this intake was estimated for males ages 12-18 years.
As shown in Table II-12, survival of *B. longum* BB536 in market products over typical shelf-life decreases and the percent survival varies by type of market product. Over the typical shelf-life, approximately 10% to nearly 100% of the *B. longum* present at the beginning of shelf-life (i.e., the time of manufacture) survive. In order to maintain the target level of 1 x 10^{10} CFU *B. longum* BB536 per serving throughout shelf-life, levels of *B. longum* BB536 approximately 1 to 10 times higher than the target level must be added at the time of manufacture, i.e. 1 x 10^{10} to 1 x 10^{11} CFU per serving. In reality, users of foods containing added *B. longum* BB536 will consume the products at various points between the beginning and end of product shelf life. Intakes of *B. longum* BB536 therefore are between the intakes estimated for the beginning and end of shelf life, or in the range of 1 to 10 times the estimated mean and 90th percentile intakes of 7.5 x 10^{10} and 1.2 x 10^{11} CFU *B. longum* BB536 for the population ages 2 years and older.

It is important to note that theses estimates are likely large overestimates of actual intakes of *B. longum* BB536 resulting from the proposed uses in the food supply. In the calculations of estimated intakes, any reported intake of a food corresponding to one of the proposed use categories (Table III-3) was assumed to contain added *B. longum* BB536. Additionally, all foods were assumed to contain the maximum proposed concentration of *B. longum* BB536 per serving. It is likely that consumers may in fact consume only a subset of these foods containing added *B. longum* BB536, and not all products may contain the maximum proposed use levels. Also, some of the proposed uses of *B. longum* BB536 may be subjected to heating during preparation (e.g., cooked breakfast cereals, toasted breads, and warmed milk beverages). The heat from some of these preparations may reduce viable counts of *B. longum* BB536.

Table III-3. Food Categories Proposed for Addition of *B. longum* BB536
Breads/baked goods
bars; includes meal replacements, high protein, snack bars
biscuits
breads/rolls (yeast); includes bagels, croissants, English muffins, pizza crust
breakfast pastries; includes Danish
cakes, includes coffee cakes
cobblers, turnovers, strudels, crisps
cookies/bars
crackers
doughnuts
pies
quick breads; includes breads, muffins, popovers, cornbread
Cereals
breakfast cereals, cooked; includes grits, oatmeal, cream of wheat, wheat cereal
breakfast cereals, ready-to-eat
Dairy products/dairy-based foods and dairy substitutes
cheese spreads
cheese, imitation
cheese, processed
cream substitutes
cream, heavy
fermented milk; includes buttermilk and kefir
flavored milk beverage mixes
frozen desserts; includes ice cream, ice milk, frozen yogurt, frozen novelties, milk shakes
follow-on infant formula
imitation milk
infant follow-on formula
meal replacements, liquids and dry mixes
milk, plain and flavored; includes cocoa, chocolate milk, fruit milks, coffee drinks (fluid/dry)
puddings and custards
smoothies
whipped toppings
yogurt
Fruit Products
juices and nectars; includes citrus, non-citrus, vegetable and blends
frozen fruit
frozen juice bars, ices
Miscellaneous
candies; includes hard candies, mints, chocolate, and all other types of confections
chewing gum
cocoa powder
condiment sauces, including catsup, BBQ, taco, steak, cocktail, Worcestershire, teriyaki, tartar
flavored beverage syrups
fruit flavored powder beverage mixes
gelatin desserts, plain or with fruit
gravies
margarine
peanut and other nut butters/spreads
snack foods; including chips, popcorn mixtures
weaning foods; includes dry cereal, snacks, juices

Table III-4. Estimated 2-Day Average Intakes of *B. longum* BB536 from Proposed Uses in Foods

Population	Users (n)	Total Survey Sample	% Users [c]	Food (servings/d)		*B. longum* BB536 (CFU/d)			
						Beginning of shelf life[a]		End of shelf life [b]	
				Mean	90th percentile	Mean	90th percentile	Mean	90th percentile
Infants < 12 mo	318	421	77	2.4	5.3	2.4E+11	5.3E+11	2.4E+10	5.3E+10
Infants 12-23 mo	279	281	100	6.4	9.2	6.4E+11	9.2E+11	6.4E+10	9.2E+10
Children 2-5 y	694	694	100	7.8	11.4	7.8E+11	1.1E+12	7.8E+10	1.1E+11
Males, 6-11 y	386	386	100	9.3	13.2	9.3E+11	1.3E+12	9.3E+10	1.3E+11
Females, 6-11 y	443	443	100	7.8	11.6	7.8E+11	1.2E+12	7.8E+10	1.2E+11
Males, 12-18 y	894	894	100	9.2	15.3	9.2E+11	1.5E+12	9.2E+10	1.5E+11
Females, 12-18 y	874	874	100	7.3	11.6	7.3E+11	1.2E+12	7.3E+10	1.2E+11
Males, 19+ y	2063	2064	100	8.0	13.1	8.0E+11	1.3E+12	8.0E+10	1.3E+11
Females, 19+ y	2297	2297	100	6.6	10.6	6.6E+11	1.1E+12	6.6E+10	1.1E+11
Total population, 2+ y	7651	7652	100	7.5	12.5	7.5E+11	1.2E+12	7.5E+10	1.2E+11

[a] Assumed a use level of 1 x 10^{10} CFU *B. longum* BB536 in each proposed use category (Table III-3) and a 10-fold overage to account for typical loss during shelf life

[b] Assumed a use level of 1 x 10^{10} CFU *B. longum* BB536 in each proposed use category (Table III-3)

[c] Weighted percent.

IV. INTENDED EFFECT

The subject of the GRAS determination, *Bifidobacterium longum* BB536, is a bacterium naturally found in the colon that contributes to the composition of the gut microflora. The intended effect of the addition of *B. longum* BB536 to foods is to provide a dietary source of this bacterium.

V. SAFETY OF *BIFIDOBACTERIUM LONGUM* BB536

A. NORMAL DEVELOPMENT AND FUNCTION OF INTESTINAL MICROFLORA

1. Development of Microflora in the Infant

At birth, the intestinal tract of a human infant is sterile. The development of infant microflora occurs in several stages: initial acquisition of microflora during birth and the first week of life, development of microflora during feeding, and changes during weaning. Various factors influence this development, including method of delivery (vaginal or Cesarean section), birth environment (home or hospital), hygienic measures in place at the time of birth, developmental stage at birth (preterm/term), type of infant feeding (breast fed versus formula fed), and the use of antimicrobials (Heavey and Rowland 1999 as cited in Rodricks et al. 2007; Orrhage and Nord 1999; Penders et al. 2006). In addition, following birth, the mother delivers additional microbial strains to the infant during suckling, kissing, and caressing (Mackie et al. 1999).

Because of these various factors, the 'normal' microflora of infants is variable. During vaginal birth, an infant is colonized by bacteria from its mother (vaginal and feces microflora) and, to a lesser extent, from the environment (Boehm et al. 2005; Dai and Walker 1999 as cited in Rodricks et al. 2007; Hammerman et al. 2004 as cited in Rodricks et al. 2007; Mackie et al. 1999). Infants delivered via Cesarean-section (C-section) often have microflora dominated by environmental/hospital isolates. Consequently, the gastrointestinal microflora of vaginally-delivered infants is quite different from infants delivered via Cesarean section (Penders et al. 2006). Infants born through C-section have lower numbers of bifidobacteria and Bacteroides, whereas they are more often colonized with *C. difficile* compared to vaginally-born infants (Penders et al. 2006).

Over the first weeks of life, facultative anaerobic bacteria begin to predominate as the oxygen in the gut is utilized and depleted (Heavey and Rowland 1999 as cited in Rodricks et al. 2007; Orrhage and Nord 1999; Conway 1997; Wold and Adlerberth 2000 as cited in Rodricks et al. 2007; Fanaro et al. 2003, Penders et al. 2006). Anaerobic bacteria including various bifidobacteria species and *Bacteroides* spp. are found as early as day 2 in the breast-fed infant's microflora, though peak counts are reached closer to day 7 (consistent with the depletion of oxygen). Other anaerobic bacteria that appear during this change include *Clostridium* spp., *Eubacterium* spp., and *Lactobacillus* spp. (Stark and Lee 1982; Lundequist et al. 1985; Balmer and Wharton 1989 as cited in Rodricks et al. 2007, Harmsen et al. 2000, Penders et al. 2006). Streptococci, enterobacteria (including *Escherichia coli),* staphylococci, and enterococci have

been identified as colonizing the infant gut one to two days after birth (Conway 1997, Lundequist et al. 1985; Balmer and Wharton 1989 as cited in Rodricks et al. 2007, Stark and Lee 1982; Balmer et al. 1989).

An important determinate of gut microflora in infants is feeding; differences have been found between the microflora of breast-fed infants and formula-fed infants. The earliest studies of the infant microflora showed that breast-fed infants had a microflora dominated by bifidobacteria, which easily out-compete other genera and the presence of which are thought to depend on the occurrence of certain glycoproteins in human breast milk (Cummings et al. 2004 as cited in Rodricks et al. 2007). In contrast, formula-fed infants have a more complex flora which resembles the adult gut in that bacteroides, clostridia, bifidobacteria, lactobacilli, Gram-positive cocci, coliforms, and other groups are all represented in fairly equal proportions (Cummings et al. 2004 as cited in Rodricks et al. 2007). Formula-fed infants have also been found to have higher fecal levels of potentially harmful bacterial metabolic by-products (Edwards and Parrett 2002).

A prospective cohort study of 1032 infants at 1 month of age used quantitative real-time polymerase chain reaction assays for the enumeration of bifidobacteria, *Escherichia coli, Clostridium difficile, Bacteroides fragilis* group, lactobacilli, and total bacterial counts in fecal samples (Penders et al. 2006). Most infants *(n* = 700) were breastfed exclusively up to the first month of life, whereas 232 infants were formula fed exclusively and 98 infants received a combination of breastfeeding and formula feeding. Exclusively formula-fed infants were more often colonized with *E coli, C. difficile, B. fragilis* group, and lactobacilli than were their exclusive breastfed counterparts (Table V-2). The counts of E *coli, C. difficile, B. fragilis* group, and lactobacilli were also significantly higher for formula-fed infants compared with breastfed infants.

In this population, 4 brands of formula were frequently used. Brand B contained locust bean gum and brand D was enriched with oligosaccharides, whereas the others were not. Infants fed exclusively with 1 of these formulas were compared. As shown in Table V-2, infants fed the oligosaccharide-enriched formula (brand D) harbored greater numbers of bifidobacteria in their stools. After adjustment for the other determinants under study, counts of bifidobacteria (coefficient: 0.60; P = .04) and also counts of lactobacilli (coefficient: 0.75; P = .02) tended to be higher for infants fed formula D, compared with reference formula A.

Table V-1. Median Counts and Prevalence of Colonization with Selected Gut Bacteria in Feces of Infants 1 Month of Age (n = 1032)

Characteristics	N	Bifidobacteria		*E coli*		*C. difficile*		*B fragilis* Group		Lactobacilli		Total Counts
		Counts, Median, log10 CFU/g Feces	Prevalence %	Counts, Median, log10 CFU/g Feces	Prevalence %	Counts, Median, log10 CFU/g Feces	Prevalence %	Counts, Median, log10 CFU/g	Prevalence %	Counts, Median, log10 CFU/g Feces	Prevalence %	Median, log $_{10}$ CFU/g Feces
Type of infant feeding												
Exclusively breastfed[a]	700	10.67	99	9.06	85	4.53	21	8.99	79	8.54	29	10.98
Exclusively formula fed	232	10.69	97	9.84	94[c]	7.43	33[c]	9.76	88[c]	8.93	41[c]	11.43[c]
Combination	98	10.78	99	9.76	93[c]	5.58	35[b]	9.53	83[b]	8.71	34	11.36[c]
Type of infant formula												
Brand A[a]	47	10.51	96	9.83	91	7.68	40	8.84	89	8.84	34	11.45
Brand B (with locust bean gum)	19	10.80	95	9.81	89	6.56	21	9.80	95	8.40	47	11.43
Brand C	39	10.81	95	9.82	93	7.28	25	9.70	87	8.68	38	11.28
Brand D (with oligosaccharides)	20	11.19	100[c]	9.63	95	6.23	30	10.11	75	9.29	55	11.65

Notes:
Source: Penders et al. 2006.
Totals may not add up to 1032 because of missing values. Counts were calculated from positive samples only.
[a] Reference category
[b] $P < .01$, as determined with the Mann-Whitney rank-sum test, calculated from all samples (the statistical significance refers to an overall difference incorporating both counts and prevalence)
[c] $P < .001$, as determined with the Mann-Whitney rank-sum test, calculated from all samples (the statistical significance refers to an overall difference incorporating both counts and prevalence)

2. Adult Microflora

The colonic microflora of infants can be described as "adult-like" after the age of two years, although populations of facultative anaerobes are often observed to be greater than those of healthy adults (Hopkins et al. 2002). Different analytical methodologies were utilized to compare the bacterial composition of feces obtained from children (16 months to 7 years), young adults (21 to 34 years), elderly subjects (67-88 years) and patients with *C. difficile* associated diarrhea (68-73 years) (CDAD) (Hopkins et al. 2002). Bacteria were determined by viable count, 16S rRNA, and community cellular fatty acids (CFA) methodologies. The results of the study suggest that gut microflora is not completely adult-like until much later in life than originally thought and probably continues to change throughout the life of an individual.

While total anaerobe counts were similar in all four subject groups, bacterial compositions at genus level varied markedly (Hopkins et al. 2002). Populations of *Bacteroides-group* organisms were significantly lower in CD AD patients compared to the other populations, while numbers of bifidobacteria were reduced in geriatric patients, irrespective of *C. difficile* infection. Viable counts of predominant bacterial species isolated from the feces of adults and children showed some variation such as higher bifidobacterial and clostridial populations in children. However, the principal microbiological difference between adults and children was the occurrence of higher numbers of enterobacteria, which were 100-fold higher in the children's feces (determined by viable counts and 16S rRNA measurements). This finding suggests that while the overall microflora composition is similar in children and adults, facultative anaerobes often remain at elevated levels in the large intestine of infants. It is probable that as the microflora matures, competition from other bacterial species increases and causes a reduction in facultative anaerobic populations. The rRNA analysis showed that the proportions of enterobacterial, bifidobacterial and *Bacteroides-group* organisms were elevated in the children, thus demonstrating that the intestinal flora of this group was bacteriologically less complex than that of the adults.

The microbiota of CD AD patients was markedly different from all other samples with greatly reduced species diversity of bifidobacteria, *Prevotella* and *Bacteroides* with a concomitant increase in clostridia and lactobacilli (Hopkins et al. 2002). Although a less sensitive diagnostic tool, marked alterations in bacterial populations detected in the CD AD patients were revealed by a greatly altered CFA profile obtained from these stools. The CFA analysis correlated with the viable bacterial counts and 16S rRNA measurements. The altered composition of metabolically active bacteria such as bacteroides and eubacteria probably leads to profound changes in the biochemical capacity of the gut microbiota with advancing age (Hopkins et al. 2002). Especially evident with aging was a reduction in the putatively protective bifidobacteria.

Once the microflora composition has become established, the major bacterial groups in the feces of adults remain relatively constant over time. It is noted, however, that elderly people have fewer bifidobacteria and higher populations of enterobacteria compared to younger adults. The frequency of isolation of *C. difficile* is also greater in the elderly. A number of physiological changes occur in the body with advancing age. These include decreased acid secretion by the gastric mucosa and greater permeability of mucosal membranes in the gut. Thus, it is likely that certain bacterial strains take advantage of new ecological niches, thereby inducing a shift in the composition of the gut microflora (Hopkins et al. 2002).

The metabolic characteristics (nine enzyme activities and 11 metabolites) of the fecal microflora were studied in children (3-15 years), adults (30-46 years) and elderly (69-89 years) subjects (Andrieux et al. 2002). The results showed large inter-individual differences in the three groups for bacterial enzyme activities. No significant differences between groups was observed for the major short-chain fatty acids (acetate, propionate, butyrate), however, the fecal concentrations of less abundant SCFA (caproate, iso-butyrate and iso-valerate) did differ among groups. Valerate, iso-butyrate and iso-valerate were significantly higher in elderly persons than in adults; they were also higher in elderly persons than in children. Ammonia concentration was significantly higher in elderly persons than in adults. The D/L lactate ratio was significantly higher in elderly persons compared to the other groups. In conclusion, data showed significant differences between elderly persons and younger adults and children, but the major metabolic characteristics of the fecal microflora were not greatly altered by the aging process.

3. Functionality of Gut Microflora

After birth the gastrointestinal tract exists in symbiosis with a large number and variety of bacteria that contribute to the health of the individual. Gut microorganisms contribute to diverse mammalian processes. Along its entirety, the microflora of the gut act as an effective barrier against opportunistic and pathogenic microorganisms. Other advantageous effects include modulation of the immune system, development of intestinal microvilli, production of short chain fatty acids (SCFA) upon which the colonic mucosa is dependent for energy, fermentation of non-digestible dietary fiber and anaerobic metabolism of peptides and proteins resulting in the recovery of metabolic energy for the host and removal of carcinogens and toxins (Rodricks et al. 2007).

The intestinal mucosa provides a natural physical cellular barrier, limiting potentially harmful microorganisms present in the intestinal lumen from colonizing enterocytes, as described earlier.

The gastrointestinal tract of the preterm neonate is physiologically immature in its development at the time of birth, rendering it more susceptible to bacterial translocation than that of the adult. Many species of pathogenic bacteria are able to alter the permeability of the intestine and invade deep tissue; these include, among others, *Salmonella* spp., *Listeria monocytogenes, Yersinia* spp., and *Shigella* spp. Infection of the intestinal epithelium often leads to diarrhea; when this fails to resolve the infection, bacteria may move into deeper tissue and eventually cause systemic infections and the related symptoms (Pucciareli et al. 1997 as cited in Rodricks et al. 2007).

Prebiotics such as galactooligosaccharides (GOS) function to increase levels of anaerobic lactic acid bacteria *(Lactobacillus acidophilus,* bifidobacteria), which have a protective role against the translocation of other bacteria. This is mediated by the production via fermentation of SCFAs that produce an acidic environment unfavorable for many pathogens, as well as production of antimicrobial bacteriocins. Bacteriocins are proteins or protein complexes with bactericidal activities directed against species that are closely related to the producer bacterium (Hammerman et al. 2004 & Dai and Walker 1999, both as cited in Rodricks et al. 2007). Several studies have investigated the enteric flora of infants with necrotizing enterocolitis (NEC) and found a decline in the concentration of anaerobic species and increased colonization with Gram-negative bacteria (Hammerman et al. 2004 as cited in Rodricks et al. 2007).

The microflora of the intestine can have significant effects on the gut-associated lypmphoid tissue (GALT). Studies examining the absence of a normal microflora demonstrated increased antigen transport across the gut mucosa (Isolauri et al. 2001). Additionally, intestinal colonization by non-pathogenic bacteria is an important antigenic stimulus for the maturation of the GALT; as the gut microflora is established, the capacity of the GALT to produce IgA secreting cells increases. The stimulatory effect of the microflora on the secretory IgA system and on B cell function in general is well established (Gaskins 1997 as cited in Rodricks et al. 2007).

B. B. Review of Bifidobacteria

The safe use of bifidobacteria in food products is supported by the long historical consumption of fermented milks and the growing knowledge about bifidobacteria taxonomy and physiology. Lactic acid-producing bacteria in foods are thought to have little or no pathogenic potential (Picard et al. 2005). Bifidobacteria are naturally present as the dominant colonic microbiota, and represent up to 25% of the cultivable fecal bacteria in adults and 80% in infants (Picard et al. 2005). Several investigators have documented the presence and variety of microbial species in

the human gut. Although this body of literature demonstrates both individual and regional differences in gut microflora, a number of *Bifidobacterium* species are commonly present in the gastrointestinal tract of both infants and adults, decreasing with age, at concentrations of 10^{10}-10^{11} and 10^{5} - 10^{8} cfu/g feces, respectively (Naidu et al. 1999). Reuter (2001) found that *Bifidobacterium* species typical for infants were *B. bifidum, B. infantis, B. breve,* and *B. parvulorum; B. bifidum* and *B. longum* could often be found in both infants and adults. Germond et al. (2002) identified *Bifidobacterium* species in Swiss adults, including *B. adolescentis, B. angulatum, B. bifidum, B. catenulatum,* and *B. longum.* Reuter (2001) found that 4 different variants of *B. adolescentis; B. bifidum* and *B. longum* could often be found in both infants and adults.

Bifidobacteria were first identified and described in the early 20th Century (Poupard et al. 1973). All known species are Gram-positive, non-motile, non-sporulating, anaerobic (although a few species can grow in air enriched with 10% CO2), catalase-negative (except *Bifidobacterium indicum* and *Bifidobacterium asteroids)* and saccharoclastic (Poupard et al. 1973; Holt et al. 1994). All species described are grouped in six different ecological niches: the human intestine, oral cavity, food, the animal gastrointestinal tract, the insect intestine and sewage (Ventura et al. 2004). Species identification is based on phenotypic and biochemical features such as cell morphology, sugar fermentation profiles and electrophoretic mobility of enzymes, constitute the first taxonomical keys used in any bacterial classification. Ambiguous results using these tools, however, prompted the introduction of DNA-DNA reassociation studies. Approximately 70% or greater DNA-DNA relatedness has been demonstrated among strains belonging to the same bacterial species. The accurate identification of many bacterial species can be accomplished by reference to rRNA gene sequences (mainly the 16S rRNA gene). All species belonging to the genus *Bifidobacterium* form a coherent phylogenetic unit, exhibiting over 93% identity among the 16S rDNA sequences found within the members of this genus (Ventura et al. 2004).

The differential characteristics of the genus *Bifidobacterium* are presented in Table V-2. Members of the this genus, like other lactic acid bacteria, produce lactic acid and acetic acid from glucose and are normal residents of the normal flora of the human intestine (Ventura et al. 2004). Morphologically they are pleomorphic and may form multiple-branching rods (bifids). The hallmark characteristic of the genus is the presence of the enzyme fructose-6-phosphate phosphoketolase which is involved in the 'bifid shunt' pathway for the metabolism of hexoses (Ventura et al. 2004).

Table V-2. Differential Characteristics of Genus *Bifidobacterium*

Characteristic	Description
Cell morphology	Irregular rods with branching; arranged singly, in pairs, in V arrangements, sometimes in chains, in palisades of parallel cells, or rosettes
Dimensions of rods	0.5-1.3 μm x 1.5-8μm
Optimum growth temperature	37-41°C
Gram Stain	Positive, but often stain irregularly
Motility	None
Anaerobic	90% or more of strains are positive
Catalase	Negative
Peptidoglycan Group[1] Diamino acid N- Glycolyl residues	 A Lysine, Ornithine Not determined
Habitat and pathogenicity	Intestines of humans and animals; sewage; pathogenicity doubtful

Notes:
Source: Holt et al. 1994. Bergey's Manual of Determinate Bacteriology.
[1] Symbolism of Schleifer and Kandler 1972 (Holt et al. 1994); group A peptidoglycans have a cross-linkage between position 3 and 4 of two peptide subunits.

Current scientific and medical literature on lactobacilli and bifidobacteria was evaluated by recognized experts on probiotics who formed a consensus on aspects of the safety of such probiotic bacteria. The experts focused on 3 key public health issues: whether probiotic consumption increases the risk of opportunistic infections due to lactobacilli or bifidobacteria, whether such probiotics that are in current use increase the potential for opportunistic infections among immunocompromised individuals and whether such probiotics are safe for consumption by young infants and children (Borriello et al. 2003). They concluded that risk of infection with probiotic bifidobacteria is similar to that of infection with commensal strains, and that consumption of such products presents a negligible risk to consumers, including immunocompromised hosts.

Bifidobacterium strains are generally held to be non-pathogenic to humans (Carr et al. 2002). There is also evidence from the literature *that Bifidobacterium* spp. lack invasive properties, i.e., the bacteria won't pass the epithelial boundary of the intestine and reach deep tissue, and that they are not mucinolytic (Zhou et al. 2000a, 2000b; 2001). These genera have been used in a variety of food products for centuries and are regularly consumed by humans on a daily basis. In addition, bifidobacteria are components of the normal flora of the human gastrointestinal tract (Ahrne et al. 1998). The lack of pathogenicity extends across all age groups and to

immunocompromised individuals (Borriello et al. 2003). *Bifidobacterium lactis* Bb12 is Generally Recognize As Safe (GRAS) for use in milk-based infant formula for infants 4 months and older, at levels not to exceed GMP. The infant formula manufacturer (Nestlé) intends to use Bb12 at levels not to exceed 10^7-10^8 cfu/g formula powder (FDA 2002).

As with any orally consumed product, there is the potential for entry into the bloodstream through cuts in the mouth, gastric lesions, or during surgical procedures. Septicemia caused by *Bifidobacterium* spp. is exceedingly rare. Lactobacilli and bifidobacteria have been estimated to account for 0.05 to 0.4 percent of cases of infective endocarditis or bacteremia (Borriello et al. 2003). Of the more than 200 reported cases in the literature, nearly all were found in a patient with an underlying, often severe, pathology (Cannon et al. 2005). There is no published evidence that consumption of probiotic foods containing bifidobacteria increase the risk of opportunistic infection among immunocompromised patients. In addition, two clinical studies have been conducted to assess the safety of probiotics in small groups of specific immunocompromised patients (e.g., patients with HIV infection) and the findings of these studies support the safety of probiotics consumed by such groups (Borriello et al. 2003).

Bifidobacteria do not appear to have the ability to translocate through the intestinal epithelial cells of mice and infect the host. Probiotics must not contain invasive characteristics, as this could result in deep tissue infections or systemic infections. During these studies, BALB/c mice were independently fed 10^{11} cfu/mouse/day for eight days. The mice were then sacrificed and their intestinal tracts were examined for villus height, crypt depth, epithelial cell height and mucosal thickness and blood, liver, spleen and mesenteric lymph node tissues were examined for viable bacterial strains. Each parameter was normal and there were no signs of infectivity or translocation to deep tissue or infection of organ systems (Zhou et al. 2000a and 2000b).

A review of publicly available literature for reports of septicemia or bacteremia caused by bifidobacteria was conducted. A total of 6 articles, citing 14 distinct cases of bifidobacteria-related bacteremia were identified. In all but two cases, patients had underlying health problems compromising their immune systems prior to infection including rectal cancer, lupus, septic pregnancy, prosthetic heart valve transplant, diabetes or chronic abdominal injury (Bourne et al. 1978, Boucaud Maitre et al. 1989, Darbas 1989, Guillard et al. 1972). In one case report, a healthy young man who had recently received invasive acupuncture therapy contracted sepsis induced by *Bifidobacterium longum* (Ha 1999). Investigators speculated that "the organism was introduced to the blood circulation either from improperly sterilized acupuncture needles or from the colon via minute perforations caused by those needles." Another case reported meningitis caused by endogenous *B. breve* in an infant born to a mother with Behçet's Disease (Hata et al.

1988). Intraveneous chloramphenicol therapy cured the patient. Although the cause of the infection was unclear, researchers noted that "it is possible that some humoral immunosuppressive factors in the mother with Behçet's Disease were transferred to the patient across the placenta." None of the reported cases identified administration of bifidobacteria as the cause of the septicemia or bacteremia.

C. EVALUATING THE SAFETY OF *B. LONGUM* BB536 INGESTION

The safe use of bifidobacteria is supported by the long historical consumption of fermented milks and the growing knowledge about bifidobacteria taxonomy and physiology (Picard et al. 2005). Despite the general safe use of bifidobacteria, some side effects in susceptible individuals are theoretically possible, including infections, deleterious metabolic activities, excessive immune stimulation, and gene transfer (Marteau and Shanahan 2003). In recognition of these potential adverse effects, FAO/WHO guidelines for the evaluation of microbes for probiotic use in foods recommend testing for several parameters (FAO/WHO 2002). Results from these assessments provide an indication of the likelihood that the microbe would cause unwanted side effects. These parameters include:

- Determination of antibiotic resistance parameters
- Assessment of certain metabolic activities (e.g., D-lactate production, bile salt deconjugation).
- If the strain under evaluation belongs to a species that is a known mammalian toxin producer, it must be tested for toxin production.
- If the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required.
- Assessment of the lack of infectivity by a probiotic strain in immunocompromised animals would add a measure of confidence in the safety of the probiotic.
- Assessment of side effects during human studies.
- Epidemiological surveillance of adverse incidents in consumers (post-market).

These parameters have been examined in *B. longum* BB536 and study findings are summarized below. Human studies of *B. longum* BB536 ingestion are summarized in section 4a of this chapter, and an assessment of side effects observed in those studies is presented in that section.

1. Antibiotic Resistance Patterns

a. Minimum Inhibitory Concentrations

Testing Conducted by Morinaga Milk Industry, Co., Ltd.

The antimicrobial resistance patterns of *B. longum* BB536 and *B. longum* ATCC15707 (the type strain) were assessed by determining the minimum inhibitory concentrations (MIC) for 28 antibiotics for each of the *B. longum* strains (Table V-3) using a broth dilution method. The resistance patterns tested included those involved in methicillin resistance, P-lactamase activity, multiple-aminoglycoside resistance, macrolide antibiotics resistance, and streptomycin resistance. An inoculum of 10^6 cells per milliliter was used. The tubes were incubated at 37°C for 24 hours under anaerobic conditions. MIC is defined as the lowest concentration of antimicrobial agent that will inhibit growth to less than 0.3 OD at 660 nm.

Results from the evaluation indicate that for 21 of the 28 antibiotics tested, the MIC of *B. longum* BB536 was less than or equal to that of the type strain, *B. longum* ATCC15707. In both strains, the highest MIC values ($\geq$ 25 ug/ml for both strains) were observed for all tested aminoglycosides (gentamicin, streptomycin sulfate, parommomycin sulfate, kanomycin and neomycin), phosphomycin disodium salt, mupirocin, rifampicin, metronidazole and polymyxin B. The MIC for polymyxin B sulfate salt was 2000 IU for both strains.

Based on the findings from the testing, *B. longum* BB536 is susceptible to clinically relevant antibiotics including selected antibiotics in the P - lactam, tetracycline and macrolide groups, as well as antibiotics belonging to other groups. Ammor and colleagues (2007) recently reviewed antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria and reported that bifidobacteria are usually very susceptible to Gram-positive spectrum antibiotics (bacitracin, erythromycin, linomucon, novobiocin, teicoplanin and vancomycin), broad-spectrum antibiotics (rifampicin, spectinomycin and chloramphenicol) and beta-lactams (penicillin, ampicillin, amoxicillin, peperacillin, ticarcillin and imipenem), though variability has been seen in their susceptibility to tetracycline, cephalothin and cefotetan. BB536 was tested for sensitivity to 9 of these 19 antibiotics reported by Ammor et al. (2007); it was sensitive to all but rifampicin.

The high MIC values for several of the antimicrobials observed for both *B. longum* BB536 and *B. longum* ATCC 15707 also are consistent with findings in other studies. Most *Bifidobacterium* species are reportedly resistant to metronidazole, neomycin, gentamicin, kanamycin, streptomycin and mupirocin (Ammor et al. 2007). Some *Bifidobacterium* strains have been considered vancomycin and cefoxitin resistant, while other strains are resistant to erythromycin,

clindamycin and tetracycline. Using a macrodilution broth method, *Bifidobacterium* species (including *B. longum)* also were found to be resistant to paramomycin sulfate (Lim et al. 1993). Masco et al. (2006) tested the susceptibility of 100 *Bifidobacterium* strains (representing 11 bifidobacterial species) from humans, animals and probiotics to 12 antimicrobial strains using agar overlay disc diffusion. Based on these results, one or two strains per species were also tested for susceptibility testing to nine antibiotics by broth microdilution using the Lactic acid bacteria Susceptibility test Medium (LSM) supplemented with cysteine. The investigators reported that bifidobacteria are intrinsically resistant to gentamicin and polymyxin B, and that all strains tested were susceptible to several antimicrobial strains including rimfapicin.

In a study of antimicrobial susceptibility of bifidobacteria, potentially acquired resistance to tetracycline and minocycline was observed in 14% of tested bifidobacterial strains (Moubareck et al. 2005). PCRs performed on the seven tetracycline-resistant strains revealed positive amplifications with the degenerative primers for *B. bifidum* R2, *B. longum* B36, and *B. pseudocatenulatum* R47. The gene responsible for tetracycline resistance in *B. pseudocatenulatum* and *B. bifidum* was identified as *tet*(W). Scott et al. (2000) identified the *tet*(W) gene in three *B. longum* strains in a fecal sample collected from a middle-aged male receiving daily tetracycline treatment over a 10 year period. The location of the *tet*(W) gene was determined to be the chromosome; transferability testing was not determined on the *B. longum* strains.

Table V-3. Antimicrobial susceptibility of *Bifidobacterium longum* BB536 and *B. longum* ATCC 15707 (Type strain)

Antibiotics	Unit	MIC	
		B. longum ATCC15707	*B. longum* BB536
β - lactam group			
Penicillin G	IU	0.78IU	0.39IU
Carbenicillin disodium salt	μ g/ml	6.25	3.13
Methicillin	μ g/ml	6.25	6.25
Ampicillin sodium salt	μ g/ml	0.78	0.78
Azlocillin sodium salt	μ g/ml	1.56	0.78
Dicloxacillin sodium salt hydrate	μ g/ml	6.25	6.25
Aminoglycoside group			
Gentamicin	μ g/ml	25	100
Streptomycin sulfate salt	μ g/ml	50	50
Paromomycin sulfate	μ g/ml	> 400	400
Kanamycin	μ g/ml	> 400	400
Neomycin	μ g/ml	200	200
Cephem group			
Cephalothin sodium salt	μ g/ml	12.5	12.5
Cephalexin hydrate	μ g/ml	12.5	25
Cefamandole	μ g/ml	3.13	6.25
Tetracycline group			
Tetracycline	μ g/ml	3.91	0.78
Doxycycline hydrochloride	μ g/ml	0.10	0.20
Peptide group			
Polymyxin B sulfate salt	IU	2000 IU	2000 IU
Bacitracin	μ g/ml	6.25	3.13
Gramicidin	μ g/ml	0.05	0.10
Macrolide group			
Erythromycin	μ g/ml	0.98	0.20
Synthetic antimicrobial group			
Metronidazole	μ g/ml	200	400
The other group			
Vancomycin	μ g/ml	0.78	0.39
Chloramphenicol	μ g/ml	1.56	3.13
Lincomycin hydrochloride	μ g/ml	0.39	0.39
Rifampicin	μ g/ml	400	200
Clindamycin hydrochloride	μ g/ml	< 0.02	0.02
Phosphomycin disodium salt	μ g/ml	800	> 800
Mupirocin	μ g/ml	> 400	> 400

Notes:
Source Morinaga Milk Industry Co., Ltd.

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Testing Conducted by PROSAFE

Morinaga Milk Industry Co. Ltd. provided the *B. longum* BB536 strain to EU-funded research project PROSAFE (Biosafety evaluation of lactic acid bacteria used for human consumption) for testing. The overall objective of the PROSAFE project was to define the criteria, standards, guidelines and regulations ensure safe use of probiotic lactic acid bacteria for humans. International efforts to develop standardized approaches for assessment of antibiotic resistance of microbes destined for food use are in progress. The concern is that non-pathogeneic, non-toxigenic microbes might serve as a source of antibiotic resistance genes that might be transferred in vivo to less innocuous members of the intestinal microbiota. The most comprehensive approach to assessing antibiotic resistance is to (1) test for phenotypic resistance, and (2) using known antibiotic resistance gene sequences, either probe total bacterial DNA for the presence of these genes or conduct genomic sequencing and look for the relevant gene sequences. In the absence of such genetic information (as is the case with *B. longum* BB536) confirming the absence of antibiotic resistance genes, the phenotypic analysis is of increased importance. The strategy recommended by the European Union ACE ART project is that phenotypic resistance for multiple strains of one species is established and the strain in question is compared to the norm for the species to determine if there are antibiotic resistances expressed outside this norm. Data on antibiotic susceptibility (testing by PROSAFE) established minimum inhibitory concentration (MIC) levels for *B. longum* BB536 and compared these to cut-off levels for multiple strains of *B. longum* on the basis of MIC distribution for 15 antibiotics. Results determined that no acquired resistances were detected for *B. longum* BB536 based on MIC comparison.

As part of the program, antibiotic susceptibility testing using completed on the submitted strains. MIC values of *B. longum* BB536 and the cut-off values for *B. longum* are shown in Table V-4. Based on these results, PROSAFE concluded that no acquired antibiotic resistance was detected in *B. longum* BB536 and the available antibiotic resistance pattern suggests that *B. longum* BB536 does not present concerns for antibiotic resistance in humans.

Table V-4. PROSAFE Antibiotic Susceptibility Testing of *Bifidobacterium longum* BB536		
Antibiotic	**MIC**	
	***B. longum* Cut-offs**	***B. longum* BB536**
Penicillin (PEN)	Inconclusive	0.25
Ampicillin (AMP)	Inconclusive	0.5
Ampicillin/sulbactam (ASU)	Inconclusive	0.5
Gentamicin (GEN)	≤128	32
Streptomycin (STR)	≤128	32
Vancomycin (VAN)	≤0.5	0.25
Tetcoplanin (TPL)	Inconclusive	≤ 0.125
Quimupristin/dalfopristin (Q/D)	≤0.032	≤ 0.032
Erythromycin (ERY)	≤0.25	0.032
Clindamycin (CLI)	Inconclusive	≤ 0.032
Oxytetracycline (OTE)	≤4	0.25
Chlorampentcol (CMP)	≤8	1
Fusidic acid (FUS)	≤32	4
Trimethoprim (TMP)	Inconclusive	128
Sulfamethoxazole/trimethoprim (SXT)	Inconclusive	32
Notes: Source August 25, 2005 report from PROSAFE to Morinaga		

b. Homology of the *B. longum* BB536 Gene with Known Antibiotic Resistant Genes

Additional testing of homology between known antibiotic resistance genes in lactic acid bacteria and the *B. longum* BB536 gene was completed by Morinaga Milk Industry Co., Ltd. The testing included 10 genes *(tet*(W), *tet*(O), *tet*(S) (Tetracycline), *erm*(B) (erythromycin), *aad*(E) (streptomycin), *mef*(A) (erythromycin), *aph*(A3) (kanamycin/streptomycin), and *aad-6* (kanamycin/streptomycin), (vatD), and (vatE) (streptogramin)). All genes were picked from the NCBI (National Center for Biotechnology Information) collection. In cases where there were two or more candidate genes in NCBI, genes from lactic acid bacteria were selected. Using BLASTN analysis, homology of the selected genes was compared with the *B. longum* BB536 gene. Results from the analysis indicate that there was no significant homology between the *B. longum* BB536 gene and the 10 selected genes from lactic acid bacteria with known antibiotic resistance (*tet*(W), *tet*(O), *tet*(S), *erm*(B), *aad*(E), *mef*(A), *aph*(A3), *aad-6,* (vatD), (vatE)). These observations suggest that *B. longum* BB536 genes do not include these antibiotic resistance genes, which are frequently observed in the genes of lactic acid bacteria. Morinaga also tested for antibiotic resistance as part of the analysis for known pathogens and toxins. This analysis is discussed in a subsequent section.

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c. Tetracycline Resistance

Results from the Morinaga testing of *B. longum* BB536 indicate that the strain is sensitive to tetracycline (Table V-3). The *B. lactis* strain that has GRAS status for use in infant formula for infants ages 4 months and older was found to bear a tetracycline resistance gene designated as the *tet*(W) gene (FDA 2005). According to information provided to FDA by Nestlé, the *B. lactis* *tet*(W) gene is chromosomally located. A single insertion sequence element located near *tet*(W) was found, which would not provide a means for interspecies transfer of *tet*(W). Nestlé presented data and information to FDA to show that the presence of a tetracycline resistance element in *B. lactis* does not present a safety problem and does not change their conclusion that the organism is GRAS for use as an ingredient in infant formula. In their response to Nestle, FDA stated, "Based on the information provided by Nestlé, as well as other information available to FDA, the agency has no questions at this time regarding Nestlé's conclusions that the presence of the *tet*(W) gene in their *B. lactis* strain does not affect the safety of the intended use of *B. lactis* as an ingredient in infant formula and that the discovery of the *tet*(W) gene in *B. lactis* does not change their previous conclusion that *B. lactis* is GRAS for its intended use as an ingredient in infant formula."

Morinaga conducted additional testing to determine if *B. longum* BB536 has tetracycline resistant genes on the chromosome. The study and results from this testing are described below. The testing was conducted on *B. longum* BB536, five strains of *B. animalis* subsp. *animalis,* seven strains of *B. animalis* subsp. *lactis,* five strains of *B. pseudolongum,* five strains of *B. thermophilum,* four strains of *B. breve,* two strains of *B. infantis,* two strains of *B. bifidum,* and the *B. longum* type strain. DNA was extracted from the liquid culture of each test organism, and Polymerase Chain Reaction (PCR) was performed using three separate primers to test for tetracycline resistance, *tet*(W), and *tet*(K), respectively.

Results from testing with the first primer showed that strains of *B. longum* BB536, *B. animalis* subsp. *animalis, B. pseudolongum, B. breve,* and *B. infantis* had no tetracycline resistance gene, while a tetracycline resistance gene was observed in strains of *B. animalis* subsp. *lactis* and *B. thermophilum.* Results from testing with the second primer indicated that *tet*(W) was not present on chromosomal DNA from *B. longum* BB536, the *B. longum* type strain, or the strains of *B. breve, B. infantis, B. bifidum,* and *B. animalis* subsp. *animalis* used in the testing. *Tet*(W) was observed, however, on all strains of *B. animalis* subsp. *lactis.* Results from testing with the third primer showed that no *tet*(K) gene was observed on the chromosome of *B. longum* BB536 or any of the other bifidobacterial strains used in the test. Overall, results from this testing provide evidence that *B. longum* BB536 does not contain a tetracycline resistant gene.

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d. Summary of Antibiotic Resistance Testing

In summary, the available information on antibiotic resistance patterns of *B. longum* BB536 indicates that the antibiotic susceptibilities of the strain are overall similar to patterns of other bifidobacterial species, the strain is resistant to clinically important antibiotics, and the strain is not likely to have transmissible antibiotic genes. These findings indicate that use of *B. longum* BB536 in foods does not present concerns for antibiotic resistance.

2. Metabolic Activities

a. D-Lactate Production

Metabolic products of both *Bifidobacterium longum* BB536 and *B. longum* ATCC 15707 (type strain) were cultured and analyzed for the presence of both D- and L- lactic acids (Table V-4). Results from this study indicate that *B. longum* BB536 produces predominantly L-lactic acid, while production of D-lactic acid is negligible.

Table V-5. Lactic Acid Production by *B. longum* BB536 and *B. longum* ATCC 15707

Strain	D-Lactic acid (mg/g)	L-Lactic acid (mg/g)	Total (mg/g)
B. longum BB536	0.07	4.00	4.07
B. longum ATCC 15707[a]	-0.05	5.67	5.67

Notes:
Source Morinaga Milk Co., Ltd Industry
Abbreviations:
[a] Type strain

b. Bile Salt Deconjugation

Grill and colleagues conducted studies (1995a and 1995b) to investigate bile salt deconjugation by bifidobacteria. In one study, Grill et al. (1995a) investigated the effect of bile salt concentrations on selected species of bifidobacteria, including *B. longum* BB536. At bile salt concentrations ranging from 0.95 to 5.5 mM, strong and rapid inhibition of growth (>80%) was observed for all bifidobacteria species, and at concentrations above 5.5 mM, complete inhibition of bacterial growth was seen. *B. longum* BB536 was observed to deconjugate 80-95% of selected bile salts. The production of deconjugated bile salts was concurrent with bacterial growth, and deconjugated bile salts were the only compound produced during bifidobacteria transformation. The investigators attributed the growth inhibition of the bile salts to production of deconjugated

bile salts.

In the other study, Grill and colleagues (1995b) investigated the ability of several species of *Bifidobacterium* to deconjugate bile salts. All tested strains, including *B. longum* BB536, were observed to deconjugate bile salts including taurocholic, taurodeoxycholic, taurochenodeoxycholic, glycocholic, glycodeoxycholic, and glycochenodeoxycholic acids. Enzyme levels increased during the growth phase. The investigators also purified and characterized conjugated bile salt hydrolase from *B. longum* BB536 and determined that the enzyme is likely a hexamer. The purified enzyme was active one glycine and taurine conjugates of cholate, deoxycholate, and chenodeoxycholate. No significant difference in the initial rate of deconjugation and enzymatic efficiency of *B. longum* BB536 was observed among bile salts. These results do not suggest a safety concern.

3. Genomic Analysis for Known Toxins and Pathogenic Markers

A full genomic analysis was completed for *B. longum* BB536. The amino acid sequences of the predicted proteins derived from the *B. longum* BB536 genomic sequence were compared to the amino acid sequences of known bacterial toxins found in the GeneBank database (release 152). The reference toxin sequences included those from *Clostridium botulinum, C. perfringens, C. difficile, Staphylococcus aureus, Bacillus cereus, Salmonella typhimurium, Escherichia coli, Shigella dysenteriae, Vibrio cholerae, C. tetani, Streptococcus pyogenes,* and several others.

Additionally, gene sequences (as amino acid sequences) of three known pathogens were compared to gene sequences of *B. longum* BB536. The gene sequences from *Pseudomonas aeruginosa* PA01 (AE004091.1), *Staphylococcus aureus N3* 15 (BA000018.3, AP003139.1), and *Clostridium perfringens* str. 13 (BA000016.3, AP003515.1) were used in the comparisons. Sequences were searched through the database of amino acid sequences expected from gene sequences of *B.longum* BB536 using BLASTP (amino acid vs. amino acid) and also using tBLASTN (amino acid vs. nucleic acid).

High homology [Morinaga: Please DEFINE what is considered to be "high homology"] between amino acid sequences in *B. longum* BB536 and the toxins and between amino acid sequences in *B. longum* BB536 and the known pathogens was not found. Results from these analyses indicate that it is unlikely that *B. longum* BB536 encodes any proteins that are similar to those used in the comparisons.

4. Hemolytic Potential

Morinaga tested the hemolytic activity of *B. longum* BB536 using BL agar plates supplemented with horse blood. The *B. longum* BB536 was diluted in a saline solution and 0.1 ml of this solution was added to the agar plate and incubated under anaerobic conditions for 72 hours. After colonization, hemolysis was not observed around any of the colonies.

5. Potential for Infectivity

It is important to determine if a probiotic microbe is able to translocate through tissue, as passage from the gastrointestinal tract to extra-intestinal tissue could lead to systemic infections. The theoretical risk for infection from bifidobacteria, however, is low (Marteau and Shanahan 2003).

Yamazaki and colleagues conducted a study to investigate bacterial translocation in gnotobiotic mice inoculated with *B. longum* BB536 (Yamazaki et al. 1985). Bacterial translocation is a phenomenon caused by a weakened intestinal barrier that results in the passage of bacteria (or bacterial components or products) across the mucous membrane and epithelium (Ishibashi and Yamazaki 2001). Given that translocation from the intestine is difficult to bring about in healthy animals, inducing techniques are often used to study translocation (Ishibashi and Yamazaki 2001). Bacterial translocation is known to occur in germ free mice; these animals therefore provide a method to study translocation.

When *B. longum* was administered orally to germ-free mice, the bacteria colonized the intestinal tract and reached of concentration of 10^9 to 10^{10} CFU/g intestinal content in 2-3 days. Translocation of the colonized *B. longum* to the mesenteric lymph nodes, liver and kidneys occurred between 1 to 2 weeks after the association. The investigators reported that the translocated *B. longum* caused neither infection nor any harmful effect. The translocated *B. longum* was observed to disappear after week 4, indicating inhibition of translocation. The phenomenon of inhibition of translocation was not observed in nude mice, and translocation reportedly did not cause infection or any harmful effect. The inhibition of translocation observed in *B.longum-monoassociated* mice is thought to be associated with T-lymphocyte-mediated immunity. The time of occurrence of translocation inhibition was observed to coincide with the time of expression of cellular immunity in the *B. longum*-monoassociated mice (Ishibashi and Yamazaki 2001).

D. REVIEW OF THE SCIENTIFIC LITERATURE ON *BIFIDOBACTERIUM LONGUM* BB536

1. Introduction to *in Vitro* and *in Vivo* Studies

Many studies have been performed with *Bifidobacterium longum* BB536. Data from these *in vitro,* animal and human studies provide information to support the safety of the use of *B. longum* BB536 in the diet.

Morinaga Milk Industry Co., Ltd. provided references to studies conducted with *B. longum* BB536. Many of these studies were originally published in Japanese and one was published in French; Morinaga provided English translations for a total of eleven of the 18 studies in humans, and 5 of the 14 studies in animals. In some of the studies the test organism is identified only as *B. longum;* Morinaga Milk Industry Co., Ltd. has indicated that the test organisms were *B. longum* BB536 unless identified as another strain of *B. longum.*

A literature search for toxicological and safety information related to ingestion of *B. longum* was conducted using the DIALOG® Toxicology and Medicine categories. Potentially relevant articles were retrieved and reviewed. Summaries of studies of *B. longum* BB536 safety are presented in Tables V-5 to V-9.

2. In vitro Studies of *B. longum* BB536

Several *in vitro* studies have been conducted to investigate properties of *B. longum* BB536 (Table V-5). Results from *in vitro* studies indicate that *B. longum* BB536 inhibits growth of pathogenic bacteria. In a co-cultivation study involving eight harmful bacterial strains including *E. coli, Klebsiella pneumoniae, Clostridium clostridiiforme, C. perfringens, Bacteroides distasonis, B. fragilis, B. thetaiotaomicron* and *B. vulgatus, B. longum* BB536 demonstrated inhibition of growth of all the strains (Araya-Kojima et al. 1995). In this study, ammonia production decreased while lactic and acetic acid production increased. In another in vitro study, *B. longum* BB536 showed antibacterial activity toward Gram-negative pathogenic bacteria including *E. coli* C1845 *and Salmonella enterica* ser. *Typhimurium* SL1344 (Makras et al. 2006). The antibacterial activity was attributed primarily to the production of acetic acid and lactic acid.

Results from other *in vitro* studies of *B. longum* BB536 indicate that acids produced by *B. longum* BB536 in cellular extracts assist in nitrite elimination (Grill et al. 1995c), and that growing *B. longum* BB536 cells are able to remove cholesterol by both precipitation and assimilation (Tahri et al. 1995).

3. Studies of *B. longum* BB536 in Animals

a. Acute, Chronic and Repeat Dose Studies

Momose et al. (1979) conducted acute toxicity tests of *B. longum* BB536 in mice and a chronic toxicity test of the organism in rats (Table V-6). In the acute studies, male and female ICR mice (10 mice/sex/group) were given a single dose of lyophilized powder containing *B. longum* either orally by syringe or intraperitoneal administration. The BB536 powder used for dosing contained 10^{12} colony forming units of bacteria per gram and was suspended in sterilized saline. For the acute oral test, mice stopped feeding 6 hours prior to administration and were given *B. longum* solution 3 times within 24 hours. The maximum doses administered orally were 53.51 g/kg-bw for males and 54.99 g/kg-bw for females (~$5x10^{13}$ CFU/kg-bw for males and females); these doses corresponded to the maximum amount of test material that was technically feasible to administer. No deaths were seen and nothing abnormal was reported during general observation or anatomical dissection in any mice given bacterial cultures orally. Since no deaths were reported at the highest dose during oral administration of *B. longum,* investigators concluded that the value of the oral LD_{50} in mice was 53.51 g/kg for males and 54.99 g/kg for females.

For the intraperitoneal administration, animals were given a single dose of solution containing *B. longum.* Mice were administered intraperitoneal concentrations of 0.30, 0.39, 0.51, 0.66 and 0.86 g/kg-bw (with a maximum concentration of ~$9x10^{11}$ CFU/kg-bw). Abnormalities reported include raised hairs, reduced voluntary movements, unsteady walking, accelerated respirations, uneasiness and closed eyes. All mice in the high dose group died on the second and third days after administration. Anatomical dissection of this group revealed almost no trace of sample inside the abdominal cavity. The researchers concluded that the LD_{50} by intraperitoneal administration in mice was 0.53 g/kg-bw for male mice and 0.56 g/kg-bw for female mice. The LD_{50} doses in the intraperitoneal study corresponds to an LD_{50} of ~$5x10^{11}$ CFU/kg-bw.

In the chronic test, the toxicity of *B. longum* was evaluated in 4 week-old SD rats (10/sex/group) given solid feed containing lyophilized bacterial powder (0.5%). A control group was given feed without lyophilized bacterial powder for comparison. Animals were given *B. longum* in feed (experimental group) or normal feed (control group) for 1 year after which results were reported.

Deaths in the control and test animals were unrelated to the administration of *B. longum* BB536 and the poor state of health of the surviving animals in this study makes interpretation of the results impossible. Therefore, it will not be described in further detail.

Several repeat-dose studies of *B. longum* BB536 administration in rats also have been conducted at calculated dosages ranging from $5.6x10^9$ to $2.4x10^{12}$ CFU/kg-bw/d in rats and 4x109 CFU/kg-bw/d in mice (Table V-6). Results from these studies of *B. longum* BB536 administration to rats show no treatment effects on body weight, body weight gain, or intake. No differences in body weight were seen in male or female F344 rats given *B. longum* BB536 in the diet at a concentration of 0.5% for up to 58 weeks (Reddy and Riverson 1993; Challa et al. 1997), 1.5% or 3% for 12 weeks (Kulkarni and Reddy 1994) or 2% for 40 weeks (Singh et al. 1997). No significant differences in body weight gain or food intake were noted in male F344 rats fed 0.5% *B. longum* BB536 in the diet until 17 weeks of age (Challa et al. 1997). The absence of changes in body weight and feed intake of tested animals is of pivitol importance, as body weight is a sensitive indicator of animal health and decreases in body weight or food intake may reflect an adverse effect of the test article. The findings of the studies provide support for the safe use of *B. longum* BB536 under the test conditions.

b. Effects on Microbial Populations and Activities

A small number of animal studies have been conducted to evaluate the effects of *Bifidobacterium longum* BB536 on microbial populations and activities in the gastrointestinal tract. Results from these studies indicate that orally administered *B. longum* BB536 is associated with elevated *B. longum* concentrations in feces from rats and mice, and that administration of *B. longum* BB536 has an inhibitory effect on pathogenic fecal bacterial concentrations in mice. No changes in fecal pH were observed. Summaries of these studies are presented in Table V-6.

In one study, C3H/He mice were given milk once daily for 6 months that contained 10^8 viable cells of *B. longum* BB536 (strain identity provided by Morinaga) and *Lactobacillus acidophilus* (Tomoda et al. 1986). During the feeding period, concentrations of *B. longum* rose from zero (at baseline) to levels in the range of 10^{10} to 10^{11} CFU per g of feces. One month after the feeding treatment ended, no *B. longum* was detected in feces. In another study in which five ovariectomized rats were fed 10^9 CFU *B. longum* BB536 daily in milk, the administered bifidobacteria was not seen in fecal samples collected at baseline or after 16 days of treatment (Igarashi et al. 1994). After 23 days of treatment, *B. longum* BB536 was detected in feces of one of the five rats; the concentration was 10^8 CFU/g. In the same study, *B. longum* was detected in feces from 80% of rats consuming *B. longum* in combination with lactulose after 16 days of treatment. On day 23 of feeing, *B. longum* was detected in feces of all rats, and the mean concentration was 10^9 CFU/g.

In rats fed a diet containing approximately 1x109 viable units *B. longum* BB536 per day for 13

weeks, no effects on cecal pH or cecal weight were seen, and diarrhea was not present (Challa et al. 1997). In another study, daily intakes of 10^9 CFUs *B. longum* BB536 in milk for 31 days had no effects on cecal pH or cecal short chain fatty acid concentrations (Igarashi et al. 1994).

Tomoda et al. (1981) studied the effect of daily administration of milk containing *B. longum* and *L. acidophilus* (approximately 10^8 CFU of each) on intestinal flora in immunocompromised mice given one of three immunosuppressive drugs (prednisolone, 5-Fu 50, or cyclophosphamide). After the 3-month treatment period, decreases in fecal concentrations of *Klebsiella, Citrobacter, Pseudomonas, P. vulgaris, Candida* and *E. coli* were seen in rats fed the supplemented milk as compared to the control group.

c. Effects on Immunity and Cancer

The effects of *B. longum* BB536 on immunity and measures of anticarcinogenesis were assessed in several studies.

Yamazaki and colleagues investigated the effects of *B. longum* BB536 inoculation on lethality of oral or intravenous exposure to *E. coli* O111 in germ free mice (Yamazaki et al. 1982.) Orally administered *E. coli* was observed to be less lethal in *B. longum*-inoculated mice as compared to germ free animals, while inoculation had a protective effect to intravenously administered *E. coli* at three weeks post-inoculation but not at 2 weeks. *B. longum* BB536-inoculated animals were also observed to have lower concentrations of endotoxin *and E. coli* in organs as compared to germ free mice following sublethal intravenous challenges.

Namba and colleagues (2003) also examined the effects of *E. coli* (O157:H7) in germ-free mice following inoculation with *B. longum* BB536. In this study, *B. longum* BB536 had an inhibitory effect on *E. coli,* as all inoculated animals survived (42 days of observation), while all mice that were not inoculation with *B. longum* BB536 died within 32 days. *E. coli* counts in fecal samples collected from the *B. longum* BB536-inoculated animals were approximately 1/10 the levels observed in the control group. Necropsy reports "some of the BB536-MA mice showed mild lesions in the kidney." No histopathology was reported by the investigators.

Other studies have been conducted to examine production of cytokines in mouse peritoneal cells (Sekine et al. 1994). Following an intraperitoneal injection of *B. longum* BB536 in male BALB/c mice, induction of inflammatory cytokine expression, including IL-ip, IL-6 and TNF-a, and also IL-10 was observed. These investigators also showed that the incidence of Meth A-induced tumors was significantly lower among mice who received *B. longum* BB536 or *B.*

animalis BB920 in combination with the subcutaneous injection of Meth A.

B. longum BB536 did not induce DNA damage (initiator activity) in hepatocytes isolated from rats receiving approximately 10^{10} or 10^{11} viable cells by gavage (Watabe 2004). Results from other studies in rats suggest that consumption of *B. longum* BB536 in the diet may provide antitumorigenic effects (Kulkarni and Reddy 1994; Singh et al. 1997; Reddy and Rivenson 1993).

4. Studies of *B. longum* BB536 Ingestion by Humans

a. Healthy Adults

Seventeen clinical studies (reported in 14 papers) involving the administration of *B. longum* BB536 to healthy adults were identified and reviewed (See Table V-7). The duration of *B. longum* BB536 consumption ranged from 6 days to 14 weeks. In three studies, intakes of *B. longum* BB536 were approximately 10^{11} CFU per day daily; participants consumed this dose for periods of 4, 13 or 14 weeks. In all other human studies we reviewed, doses were in the range of approximately 10^{9} to 10^{10} CFU *B. longum* BB536 per day. In all but one study (Ballongue et al. 1993), *B. longum* BB536 was administered once per day. None of the studies reported any participant dropouts or adverse events due to the test articles.

In a study that included participant questionnaires as part of protocol, no subjects reported discomfort or side effects related to the intake of approximately 10^{10} CFU *B. longum* BB536 (Tomoda et al. 1990, 1991). No incidents of constipation or diarrhea were reported nor were any changes in appetite or bodyweight identified over the 6-week test periods. Moreover, laboratory tests demonstrated no change in blood lipids, proteins or liver function tests in the study participants (Tomoda et al. 1990, 1991).

In addition to providing information that supports the safe and well-tolerated use of *B. longum* BB536, several of the studies in healthy adults assessed the impact of the organism on gut microbiota and activities. Ballongue et al. (1993) studied the effects of fermented milk containing *B. longum* BB536 on fecal microflora in healthy adults in two separate feeding studies. Each study began with a 2-week washout period, baseline samples were collected during the next two weeks, and then study treatments were initiated. In each study subjects consumed a total of 3.75×10^{9} CFU *B. longum* BB536, as provided in three doses each day in milk. After three weeks of treatment in the first study, fecal concentrations of total anaerobes and bifidobacteria were higher (increases of approximately 2 and 4 log units, respectively) in the

12 adults in the test group as compared to concentrations at baseline or in the 12 adults who did not consume fermented milk. At the end of the feeding period, subjects in the *B. longum* BB536 group also had decreased fecal concentrations of clostridia, bacteroides, and *E. coli* (decreases of approximately 2 to 4 log units) as compared to baseline values or controls. Fecal samples collected from subjects in the *B. longum* BB536 group three weeks after the end of the treatment were found to have concentrations of bifidobacteria that were lower than those found at the end of the feeding period, but higher than baseline levels; levels of fecal anaerobes, clostridia, bacteroides and *E. coli* increased, but remained below baseline levels.

In the second feeding study reported by Ballongue et al. (1993), healthy adults (9 per group) consumed fermented milk containing *B. longum* BB536 or a tagged version referred to as BB536 S15; subjects in the control group consumed a standard yogurt. The effects of *B. longum* BB536 and BB536 S15 on fecal flora observed in this phase were similar to the results observed in the earlier phase. Analysis of fecal samples for BB536 S15 indicated that the strain accounted for 80% of the bifidobacteria population at the end of the 3-week treatment period and 60% at measurements taken at the 3-week follow-up. It is important to note that it is not possible to determine if any of the differences across groups or over time were statistically significant, as no statistics were presented in the study. Results from these studies do indicate, however, that *B. longum* BB536 survives in the human gastrointestinal tract, and results suggest that *B. longum* may influence desirable shifts in the microflora.

Ogata and colleagues (1997) examined the effects of *B. longum* BB536 consumption on fecal flora, putrefactive substances and enzyme activities in 12 healthy adults. After one week of daily consumption of milk containing $2x10^9$ or $2x10^{10}$ CFU *B. longum* BB536 (7 and 5 subjects, respectively), no significant changes in fecal microflora were observed with the exception of a decrease in *Clostridium* (other than *C. perfringens)* in the low dose group. Additionally, no changes in relative percentages of selected bacterial groups were measured. Fecal samples collected from adults consuming the high dose of *B. longum* BB536 ($2x10^{10}$ CFU) daily were found to have lower concentrations of ammonia and beta-glucuronidase as compared to the control period, and adults consuming the low dose of *B. longum* BB536 ($2x10^9$ CFU) had lower concentrations of fecal tryptophanase. The treatments had no effects on fecal pH, total organic acids or acetic acid, though the percent of fecal water increased above control period in the high dose group (82.3% vs. 76.5%, respectively).

Several studies were conducted to assess the effects of yogurt with added *B. longum* BB536 on fecal microbial counts and activities. Active cultures in the yogurts included *S. thermophilus* with or without *L. delbrueckii* subsp. *bulgaricus.* Concentrations of bifidobacteria in fecal

samples from three adults fed yogurt containing more than $1.3x10^{10}$ CFU *B. longum* BB536 for 6 weeks were higher after treatment as compared to baseline levels, and fecal ammonia concentrations decreased (Tomoda et al. 1990, 1991). Daily intake of yogurt with $2x10^9$ viable cells of *B. longum* BB536 for two weeks resulted in significant increases in both the number and relative percentage of bifidobacteria as compared to the control period (Yaeshima et al. 1997). Yaeshima et al (1997 and 1998) also reported increases in stool frequencies of women consuming yogurt with added *B. longum* BB536. In a study of 6 healthy adults (Ogata et al. 1999), daily intake of yogurt supplemented with more than 5×10^9 viable cells of *B. longum* BB536 resulted in a significant increase in fecal *Lactobacillus* counts compared to levels before the intervention or during consumption of standard yogurt (log 7.1 vs. log 5.7 and log 5.1) and a significant increase in the proportion of *Bifidobacterium* as compared to levels before and after the treatment (27.6% vs. 20.2% and 21.0%). No other changes in counts or relative proportions of fecal microbial populations were found. The investigators speculated that the high baseline count *of Bifidobacterium* (mean value of log 9.8) may have reduced the impact the *B. longum* BB536 may have had on this test population.

Effects of *B. longum* on frequency of defecation in constipated adults were assessed. In the second part of the study by Ogata et al. (1997), administration of $2x10^9$ CFU *B. longum* BB536 in milk one time per day to 40 young constipated women resulted in a significant increase in defecation frequency and stool softness over the course of the 3-week treatment period. In another study, 18 constipated elderly adults consumed milk containing $2x10^{10}$ viable cells of both *B. longum* BB536 and *L. acidophilus;* after 10 days of treatment, a significant increase in stool frequency was observed as compared to baseline and the control periods (Seki et al. 1978). Hospitalized pregnant women consuming a yogurt prepared with *B. longum* BB536, lactulose and lactic acid bacteria reported improvements in subjective measures of gastrointestinal health defecation frequency (Ebisawa et al. 1985).

One study, for which only the abstract was available, investigated the prophylactic effects of *B. longum* BB536 on influenza virus infections in elderly adults (Nanba 2006). Test subjects received the influenza vaccination and were subsequently administered one sachet of BB536 powder containing 10^{11} CFU BB536 daily for 14 weeks. The investigators reported a significant decrease in the number of subjects that contracted influenza, developed fevers and used antibiotics during the 14 week test period.

b. Compromised Adults and Children

Eight clinical studies involving the administration of *B. longum* BB536 to unhealthy adults or

children were identified and reviewed (See Table V-8). Patient populations included adults with *H. pylori* infections (De Vrese et al. 2001), leukemia (Kageyama et al. 1984 and 1987, Sekine et al. 1985, Tomoda et al. 1981, 1986 and 1988), non-Hodgkin's lymphoma or malignant teratoma (Sekine et al. 1985) solid cancers including esophageal, stomach and lung cancer (Tomoda et al. 1981), and ulcerative colitis (Inoue et al. 2006). Study durations ranged from 8 weeks to 1 year. In all studies *B. longum* BB536 was administered once a day with the exception De Vrese et al. (2001) in which *B. longum* BB536 was administered twice a day. Daily viable cell intakes for adults were in the range of 10^9 to 10^{10} CFU in most studies, and approximately 10^{11} CFU *B. longum* BB536 per day in one study. Daily doses of *B. longum* BB536 in populations of children were approximately 10^9 CFU.

None of the studies reviewed reported adverse events or patient dropouts as a result of *B. longum* BB536 supplementation. De Vrese et al. (2001) reported that patients consuming $2.5x10^{10}$ CFU *B. longum* BB536 in yogurt experienced less diarrhea and fewer gastrointestinal distresses during and following antibiotic treatment as compared to subjects consuming a control yogurt. Other studies reported a trend in the decreased proliferation of harmful bacteria typically seen during administration of anticancer therapies or immunosuppressive drugs (Kageyama et al. 1984; Tomoda et al. 1981), or an increase in *Bifidobacterium* and *L. acidophilus* levels (Kageyama et al. 1987). In the 1984 study by Kageyama et al., the investigators also observed a decrease in the incidence of positive urine indican and blood endotoxin tests when *B. longum* BB536 was added to an antileukemic drug therapy regimen. Tomoda et al. (1981) studied 60 cancer patients; a trend in decreased fecal concentrations *of Klebsiella, Citrobacter, Pseudomonas, P. vulgaris* and *Candida* was observed during the *B. longum* treatment.

Sekine et al. (1985) assessed the effect of *B. longum* BB536 administered in milk over the course of a year on the chemiluminescence (CL) reaction of peripheral leukocytes and mean corpuscular volume (MCV) of red blood cells in eight children with leukemia. CL reactions were enhanced during the treatment. The investigators speculated that this effect was due to activation of monocyte-macrophage cell populations. Decreased red blood cell mean corpuscular volumes also were seen (89.3 fl before dosing vs. 86.0 fl after dosing); this decrease was attributed to folic acid in the milk treatment.

In another clinical observational study involving three patients with chronic hematological diseases, Tomoda et al. (1986) observed that within three months of daily supplementation with milk containing 10^9 CFUs of *B. longum* and *L. acidophilus,* fecal concentrations of *B. longum* rose from levels below the limit of detection to 10^7 to 10^9 CFU per g feces. An increase in the endogenous microbe *Bifidobacterium adolescentis* was also observed.

c. Infants

Two studies involving the administration of *B. longum* BB536 to infants were identified and reviewed (Table V-9). Akiyama et al. (1994) studied preterm infants. These subjects received $5x10^8$ CFU of *B. longum* administered via formula daily for 8 weeks. Bennet et al. (1992) studied term infants over a 5-day period, administering $9x10^9$ CFU/day BB536 to infants who had just been taken off of postnatal antibiotic treatment. Neither study reported adverse events due to *B. longum* BB536 supplementation.

Akiyama et al. (1994) conducted a randomized, controlled trial on 10 preterm infants who were exclusively breast fed during the 8 weeks of the study. Five infants were in the *B. longum* BB536 treatment group. The organism was administered in water to infants once daily. Fecal collections were made at weeks 1, 2, 4, 6, 8 and 12 of the study. All infants appeared to respond differently to the supplementation. In one of the infants, *B. longum* BB536 was detected at all collection points. In another infant, the organism was only detected up to the 6^{th} week of treatment. In a third infant, *B. longum* BB536 was never detected in the feces. In the two remaining infants, *B. longum* BB536 was only identified at one collection point. Through the use of carbohydrate utilization profiling, the investigators determined that the *B. longum* isolated from the fecal samples of the infants was *B. longum* BB536, the administered strain. *B. breve* was the dominant strain in fecal samples collected from infants in the control group; this strain was identified by the investigators as being the predominant strain among infants in the neonatal intensive care unit of the hospital used.

d. Conclusions

Studies of the effects of *B. longum* BB536 on humans were identified and reviewed. Reports of side-effects from study participants and overall tolerance of the organism in these studies can be used to assess the safety of *B. longum* BB536 in foods. Studies involving the administration of *B. longum* BB536 to healthy adults and immunocompromised individuals or adults with other severe health concerns suggest that consumption of the organism at doses in the range of 10^9 to 10^{11} CFUs is well tolerated and does not result in any side effects or safety concerns. Additionally, no adverse effects were observed in populations of infants consuming *B. longum* BB536.

Most of these studies assessed effects of the organism on microflora populations and activities, and most studies employed a crossover design in which each subject served as his or her own

control. Many studies involved small numbers of subjects, were not randomized, and did not present statistical comparisons. Given these limitations, it is difficult to draw definitive conclusions regarding the probiotic properties of the organism. Consumption of *B. longum* BB536 resulted in favorable shifts in microbial populations in some though not all studies, and results from some studies suggest that frequency of defecation may be positively altered during consumption of *B. longum* BB536.

E. REVIEW OF SCIENTIFIC LITERATURE ON OTHER STRAINS OF *B. LONGUM*

Research on bifidobacteria has been conducted on several strains of *Bifidobacterium longum,* and results from studies of other strains of *B. longum* provide corroborative evidence for the safety of human consumption of *B. longum* BB536. Two studies were identified in which two different strains of *B. longum* were fed to animals, and one study of administration of a strain of *B. longum* to adults was identified. The animal studies are summarized in Table V-10, and the human study is summarized in Table V-1 1.

1. Animal Studies

Bifidobacterium longum SPM1205 extracted from healthy adult Korean volunteers was administered to male Sprague-Dawley rats (Choi et al. 2005). The study consisted of three groups with a total of 18 rats, 6 per group. The groups included an SPM1205 test group, a probiotic cocktail containing *L. casei, L. rhamnosus, L. lactic, L. plantarum,* and *B. longum* (strain not specified) as a "commercial reference" group and a control-group in which rats were administered sterile phosphate buffer (pH 6.8). All rats were orally inoculated with 0.3 ml of their respective test-substance daily for 4 weeks. The *B. longum* treatment group received the equivalent of $1x10^9$ CFU/kg-bw/day of *B. longum* SP1205. Test rats were acclimated for one week after arrival prior to test-substance administration at 5 weeks of age. Rats demonstrating optimal growth were selected for the study and randomly assigned to one of the three groups. Animals were housed individually for the entire experiment. Animals were supplied food and water *ad libitum.* Feed intakes and body weight were monitored periodically throughout the experiment. Blood samples were collected via cardiac puncture and collected into EDTA non-treated tubes for analysis at the end of the 4-week treatment period. The gross anatomy of visceral organs was inspected and recorded for each rat. The weight of each organ was compared between test and control groups. Fecal samples were also taken from rats to compare the number of *Bifidobacterium* spp. between treatment and control rats. All statistical analyses for comparison were performed using SAS software.

All animals appeared clinically healthy and no noticeable abnormal behavior, changes in activity, or decline in hair luster was observed at the end of the treatment period. Weekly live body weight gain measurements revealed that growth rates were not affected by the administration of *B. longum* SPM1205. No differences in specific growth rate or patterns were observed between the treatment groups and the control group. Fecal samples revealed the presence of 100-times more *Bifidobacterium* spp. in the treatment-groups compared to the control. Moreover, inhibition of harmful enzymes including P-glucosidase, P-glucuronidase, tryptophanse and urease were potently inhibited by the administration of *B. longum* SPM1205.

Biochemical assays revealed that the metabolism of protein, carbohydrates and lipids were not affected by the test strain. Upon necropsy, macroscopic observation revealed no obvious differences in the size or appearance of visceral organs among groups. No hepatomegally or splenomegally enlargements were observed.

The investigators concluded that feeding rats with *B. longum* SP1205 isolated from healthy Koreans for 4 weeks had no adverse effects on the animals' general health status, growth, blood biochemistry or histological parameters.

In a 7-day restricted diet study on 20 ICR mice, *B. longum* OLL6001 resulted in no body weight change (Hidemura et al. 2003).

2. Human Study

A randomized, double-blinded, controlled parallel study was conducted on 39 healthy Finnish volunteers (Makelainen et al. 2003). Volunteers were 19 to 60 years old. Over a period of 5 weeks, subjects were given a total of $2x10^9$ CFU *B. longum* 46 and *B. longum* 2C two times per day in capsulated form. No differences in intestinal complaints were reported between groups, nor was there any change in complaints during the consumption of the bifidobacteria. The two strains of *B. longum* were well-tolerated. The investigators concluded that the *B. longum* test strains were safe for testing in elderly subjects who tend to have more compromised gut health. In another study involving 12 healthy volunteers with an average age of 22.9 years old, no safety-related adverse events were reported by investigators after the consumption of $>10^9$ CFU *B. longum* for three weeks.

F. CORROBORATIVE EVIDENCE FOR THE SAFETY OF *BIFIDOBACTERIUM LONGUM* BB536: ANIMAL TOXICITY STUDIES USING OTHER SPECIES OF BIFIDOBACTERIA

1. Introduction

While other bifidobacterial strains are not identical to *B. longum* BB536, they share many common characteristics. Therefore, studies on other bifidobacteria species commonly used as probiotics or in food production, such as *B. breve* and *B. infantis,* provide corroborative evidence to support the evidence that *B. longum* BB536 is safe for human consumption.

Acute and subchronic studies on *Bifidobacterium breve* and *Bifidobacterium infantis* are reviewed below and summarized in Table V-12. Results from both acute studies in rats demonstrate that under conditions of the tests, neither *B. breve* nor *B. infantis* present toxicological concerns at the doses tested. Results from the subchronic toxicity of *B. infantis* and *B. breve* in rats demonstrate that under conditions of the tests, No Observed Adverse Effect Levels (NOAELs) were estimated to be above the doses tested. Ikeya (2005a) reported a NOAEL of over 1,000 mg/kg-bw/day powder ($7.6x10^{10}$ CFU *B. infantis/kg-bw/day)* in rats consuming *Bifidobacterium infantis* M-63 powder (a proprietary mix made by Morinaga Milk). In this study, decreases in absolute and relative weights of the seminal vesicle in test-group males and relative weights of the spleen in test-group females were observed; since histopathological examination revealed no abnormalities and the degree of changes was slight, the investigator determined these changes "to be of no toxicological significance." The same investigator reported *Bifidobacterium breve* M-16V powder, another proprietary mix made by Morinaga Milk, to have a NOAEL of over 1,000 mg/kg-bw/day powder ($2.3x10^{1}1$ CFU B. breve/kg-bw/day) in rats (Ikeya 2005b). In this study, hematological examination revealed higher mean corpuscular hemoglobin (MCH) in test-group males; no changes in other mean corpuscular indices or erythrocyte examination caused the observation to be judged as incidental. Blood chemistry examination revealed higher total bilirubin in test-group males. Again, the degree of changes was slight and histopathological examination revealed no abnormalities; findings were again judged to be incidental. Results from these studies provide corroborative evidence of the safety of bifidobacteria, including *B. longum* BB536.

2. *Bifidobacterium breve*

a. Acute Study

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Bifidobacterium breve M-16V powder was administered to two male and two female rats at a dosage level of either 3,000 mg/kg-bw powder or 6,000 mg/kg-bw powder in a preliminary study (Onishi 1992). Because a dose of 6,000 mg/kg-bw was found to be acceptable for both male and

female rats, it was defined as a high dose; 3,000 mg/kg-bw powder was defined as the low dose for the experiment. The study was conducted by Fukushima Laboratory, Medical Scientific Research Laboratory Co., Ltd. Results from this study have not been published.

A total of 30 male and 30 female Crj :CD (SD) rats were used in the study. Rats underwent a 7-day acclamation period prior to administration of the test substance. The animals were allocated into three groups: a 6,000 mg/kg-bw powder treatment group, a 3,000 mg/kg-bw powder treatment group, and a solvent-control group. For *B. breve* M-16V, the viable cell count must be more than $1.0x10^{11}$ per gram. Ten males and ten females were allocated to each group. Animals were 3-weeks old at the time of test-substance administration. Animal body weights were measured at the time of allocation and compared with that obtained at the end of the acclimatization period. The animals were arranged in order of those whose body weight and variance was equal throughout the groups using both random number and turnover methods (Onishi 1992). No more than five animals were allotted to each laboratory cage. The animals were fed radiation-sterilized pellets and tap water containing Sodium hypochlorite. Water was provided *ad libitum* from an automatic water supply.

All animals were fasted from the evening of the day before administration. Animals were force-fed their respective treatments via a metal gastric tube. Animals were observed for any clinical abnormalities for 14 days. Observation began immediately after administration and was conducted frequently up to 5 hours post-administration. Thereafter, animals were evaluated once daily. The body weight of all surviving animals was measured on day 0 (prior to administration), 2, 4, 6, 8, 10, 12, and 14.

No cases of death were related to the administration of the test substance. No abnormal signs were observed during the 14-day observation period. Body weight for males in the 6,000 mg/kg-bw group was smaller than that of the 3,000 mg/kg-bw group and the control. According to the Student's t-test, between days 8 and 10 of observation, this difference in body weight became significant. By days 12 to 14 the difference had disappeared. These changes were not considered to be attributable to the test substance. For female rats, changes in body weight in both the 6,000 mg/kg-bw powder and 3,000 mg/kg-bw powder groups were equivalent to those in the control group.

All animals were necropsied at the end of the 14-day observation period. The following organs were observed: the brain, eyeballs, submanibular gland, hypophysis, thyroid gland, trachea, esophagus, thymus, lungs, heart, liver, spleen, pancrease, kidneys, adrenal gland, stomach, duodenum, small intestine, cecum, large intestine, rectum, mesenteric lymph nodes, bladder,

testes, epididymis, ovary and uterus. No abnormal findings attributable to the test substance were observed. The investigator concluded that one-time gastric administration of a maximum of 6,000 mg/kg-bw *B. breve* powder was not toxic. According to product specifications, this equates to a minimum of $6.0x10^{11}$ CFU/kg-bwB. *breve.*

b. Subchronic Study

Bifidobacterium breve M-16V powder was administered to groups of male and female Sprague-Dawley strain SPF (Crj:CD(SD)IGS) rats (10 rats/sex/group) by oral gavage at powder dose levels of either 0 (saline added cornstarch as control) or 1,000 mg/kg-bw/day for 91 days (Ikeya 2005b). The number of viable bacteria in the powder was recorded as being $2.3x10^{1}1$ per gram, equivalent to $2.3x10^{1}1$ CFU B. breve/kg-bw/day. This study was conducted by Bozo Research Center Inc.; results from the study have not been published. The dose level was selected based on the results of a previous 2-week oral toxicity in which M-16V powder was administered at dosages of 250, 500 or 1,000 mg/kg-bw/day. No clear toxicological changes were associated with any of the dosages; the highest dose from this study was selected as the administration dose for the current study.

The powder was weighed and suspended in physiological saline to the appropriate concentration. The control-group cornstarch was prepared in the same manner. Test rats were acclimated for eight days after arrival prior to test-substance administration at 6 weeks of age. Twenty males and 20 females showing normal body weight gain and no clinical or ophthalmological abnormalities were selected for the study. Selected animals were ranked according to body weight and assigned to each group in such a way that mean group body weight was comparable among groups. Animals were assigned to groups by a combination of the block placement method and a random sampling method using a computer. Animals were housed individually for the entire experiment. Animals were fed a pelleted CFR-1 diet and tap water. Food and water were provided *ad libitum.* Body weights of all experimental animals were measured three times, on days 1, 4 and 7 during week 1 of administration and twice a week every 3 to 4 days thereafter. Body weights were always taken prior to dosing. On the day of necropsy, animals were weighed after 16 hours of fasting in order to calculate relative organ weight. The amount of food consumed was calculated on a per day per animal basis using one day consumption measurements or three- and two-day cumulative measurements each week.

All animals were clinically observed for external appearance, nutritional condition, posture, behavior and any abnormalities 3 times a day before dosing, immediately after and approximately 2 hours after dosing each day. On weekends and holidays, animals were

evaluated twice daily (prior to and immediately after dosing).

Ophthalmological examinations were conducted twice: once during the acclimation period and once in week 13 of administration. The cornea, eye chamber, lens, vitreous body and fundus oculi of all animals were observed using a direct ophthalmoscope. Urinalysis was also done in week 13 of the experiment. After dosing on the final day of test-substance administration, all animals were individually placed in cages equipped with a urine collector. Four-hour urine was collected under deprivation of food but with free access to water; 20-hour urine with free access to food and water was also collected. Urine volume was calculated by totaling the amount of 4-hour and 20-hour urine. Urine pH, protein, ketones, glucose, occult blood, bilirubin, urobilinogen, color, sediment, volume, osmolality, sodium, potassium and chloride were analyzed. Hematological and blood chemistry examinations were evaluated in blood samples collected at necropsy from the abdominal aorta. All animals survived until termination and all organs and tissues including those in the cephalic, thoracic and abdominal regions were examined. Absolute and relative weights (calculated on a per 100g body weight basis) of animal organs were obtained from the brain, pituitary, thyroid gland (including parathyroid gland), adrenal, thymus, spleen, heart, lung (including bronchial tubes), salivary glands (including submanibular gland and sublingual gland), liver, kidney, testis, prostate, seminal vesicle, ovary and uterus.

Body weight data, food and water intake, quantitative data of urinalysis, hematological results, blood chemistry values and organ weights were analyzed for homogeneity of variance for each group using the F-test. The difference of mean values between the control group and each test group was analyzed by the Student's t-test. The difference of mean ranks between the control group and the test groups was analyzed by the Aspin-Welch t-test.

General conditions and behavior were not adversely affected by the test substance in any group. No significant body weight differences were observed between the test and control groups for either males or females. Food consumption was also comparable to that of the control, with no significant differences occurring. No significant changes were observed in any organs in the male or female rats in the treatment group. Ophthalmoscopic examination revealed no abnormalities. Urinalysis, hematological examinations, blood chemistry examinations and histopathological examinations were determined to reveal no treatment-related changes in male or female rats.

Gross pathological findings resulted in no test substance-related observations. During hematological examination, mean corpuscular hemoglobin (MCH) was found to be significantly

higher in treatment-group males compared to the control group. Hematological parameters measured included: red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte percentage, platelet count, white blood cell count (WBC), differential white blood cell count, prothrombin time, activated partial thromboplastin time (APTT) and fibrinogen). Since no changes in other mean corpuscular indices or erythrocyte examination were observed, the increase in male MCH was judged to be incidental. Blood chemistry parameters measured included AIP, total cholesterol, triglyceride, phospholipids, total bilirubin, glucose, blood urea nitrogen, creatinine, sodium, potassium, chloride, calcium, inorganic phosphourus, total protein, albumin and A/G ratio. Total bilirubin was found to be significantly higher in treatment-group males. Since the degree of the changes was slight and histopathological examination revealed no abnormalities, this finding was again recorded as being incidental.

No treatment-related abnormalities were found during the gross necropsy examination nor were treatment-related microscopic changes noted in any of the organs or tissues examined. All changes seen were considered to be incidental. The investigators concluded that oral intake of 1,000 mg/kg-bw/day of *Bifidobacterium breve* M-16V powder ($2.3x10^{11}$ CFU/kg-bw/day) for 91 days occurred without signs of toxicity. The NOAEL for this study was estimated to be above 1,000 mg/kg-bw/day *B. infantis* powder, the highest dose tested.

3. *Bifidobacterium infantis*

a. Acute Study

Bifidobacterium infantis M-63 powder was administered in a suspension of disinfected saline solution to a group of 5 male and 5 female Crj :CD (SD) IGS (SPF) rats once via a stomach tube at a dosage of 4,000 mg/kg-bw powder, or approximately 3.2×10^{11} CFU/kg-bw (Onishi 2001). According to product specifications, the viable cell count in the powder must be more than $8.0x10^{10}$ per gram. After administration, the animals were observed for two weeks, after which all animals underwent necropsy. The study was conducted by Mitsubishi Chemical Safety Institute Ltd; results from this study have not been published. Test animals were quarantined and acclimated for 5 days prior to the test substance administration. All rats were confirmed to be healthy. On the day of administration, rats were 5 weeks old. Animals were fasted for approximately 18 hours prior to test-substance administration. The diet was withheld for an additional three hours after administration. Animals were housed five to a cage, separated by sex, for the entire experiment. Food and water were fed *ad libitum* and renewed weekly. Body

weights were measured just before administration and on Days 4, 8 and 15 of observation. The clinical condition and behavior of the animals were observed at 1, 3 and 6 hours after administration. Thereafter, daily evaluations were conducted for 14 days.

All animals survived until scheduled sacrifice. General conditions and behavior were not adversely affected by the test substance in any of the animals. The body weights of all animals reportedly increased at a normal rate. No abnormalities were found in any of the animals at necropsy. According to the researcher, no histopathological examinations were conducted due to the absence of abnormalities at necropsy. The investigator concluded that one-time acute gastric administration of 4,000 mg/kg-bw *B. infantis* powder was well tolerated. The LDLo for this study was determined to be greater than 4,000 mg/kg-bw powder, the highest dose tested, which is equivalent to approximately $3.2x10^{1}1$ CFU B. *infantis/kg-bw.*

b. Subchronic Study

Bifidobacterium infantis M-63 powder was administered to groups of male and female Sprague-Dawley strain SPF (Crj:CD(SD)IGS) rats (10 rats/sex/group) by oral gavage at powder dose levels of either 0 (saline added cornstarch as control) or 1,000 mg/kg-bw/day for 91 days (Ikeya 2005a). The number of viable bacteria in the powder was recorded as being $7.6x10^{10}$ per gram, equivalent to $7.6x10^{10}$ *CFUB. infantis/kg-bw/day.* This study was conducted by Bozo Research Center Inc.; results from the study have not been published. The powder was weighed and suspended in physiological saline to the appropriate concentration.

The control-group cornstarch was prepared in the same manner. Test rats were acclimated for eight days after arrival prior to test-substance administration at 6 weeks of age. Twenty males and 20 females showing normal body weight gain and no clinical or ophthalmological abnormalities were selected for the study. Selected animals were ranked according to body weight and assigned to each group in such a way that mean group body weight was comparable among groups. Animals were assigned to groups by a combination of the block placement method and a random sampling method using a computer. Animals were housed individually for the entire experiment. Animals were fed a pelleted CFR-1 diet and tap water. Food and water were provided *ad libitum.* Body weights of all experimental animals were measured three times, on days 1, 4 and 7 during week 1 of administration and twice a week every 3 to 4 days thereafter. Body weights were always taken prior to dosing. On the day of necropsy, animals were weighed after 16 hours of fasting in order to calculate relative organ weight. The amount of food consumed was calculated on a per day per animal basis using one day consumption measurements or three- and two-day cumulative measurements each week.

All animals were clinically observed for external appearance, nutritional condition, posture, behavior and any abnormalities 3 times a day before dosing, immediately after and approximately 2 hours after dosing each day. On weekends and holidays, animals were evaluated twice daily (prior to and immediately after dosing).

Ophthalmological examinations were conducted twice: once during the acclimation period and once in week 13 of administration. The cornea, eye chamber, lens, vitreous body and fundus oculi of all animals were observed using a direct ophthalmoscope. Urinalysis was also done in week 13 of the experiment. After dosing on the final day of test-substance administration, all animals were individually placed in cages equipped with a urine collector. Four-hour urine was collected under deprivation of food but with free access to water; 20-hour urine with free access to food and water was also collected. Urine pH, protein, ketones, glucose, occult blood, bilirubin, urobilinogen, color, sediment, volume, osmolality, sodium, potassium and chloride were analyzed. Hematological and blood chemistry examinations were evaluated in blood samples collected at necropsy from the abdominal aorta. All animals survived until termination and all organs and tissues including those in the cephalic, thoracic and abdominal regions were examined. Selected organs and tissues were removed and absolute and relative weights (calculated on a per 100g body weight basis) were taken.

Body weight data, food and water intake, quantitative data of urinalysis, hematological results, blood chemistry values and organ weights were analyzed for homogeneity of variance for each group using the F-test. The difference of mean values between the control group and each testgroup was analyzed by the Student's t-test. The difference of mean ranks between the control group and the test groups was analyzed by the Aspin-Welch t-test.

General conditions and behavior were not adversely affected by the test substance in any group. No significant body weight differences were observed between the test and control groups for either males or females. Food consumption was also comparable to that of the control, with no significant differences occurring. Ophthalmoscopic examination revealed no abnormalities. Urinalysis, hematological examinations, blood chemistry examinations and histopathological examinations were determined to reveal no treatment-related changes in male or female rats.

Significant decreases were observed in the absolute and relative weights of seminal vesicles in males and relative weights of the spleen in females in the test-groups. Histopathological examination revealed no abnormalities and the changes observed were slight. The changes were determined to be incidental and were not considered to be toxicologically significant. No other

significant effects on absolute or relative organ weights were seen in the brain, pituitary, thyroid gland (including parathyroid gland), adrenal, thymus, spleen, heart, lung (including bronchial tubes), salivary glands (including submanibular gland and sublingual gland), liver, kidney, testis, prostate, seminal vesicle, ovary or uterus.

No changes in hematological parameters (red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte percentage, platelet count, white blood cell count (WBC), differential white blood cell count, prothrombin time, activated partial thromboplastin time (APTT), or fibrinogen) were seen. No changes in blood chemistry measures were observed. The parameters measured included AIP, total cholesterol, triglyceride, phospholipids, total bilirubin, glucose, blood urea nitrogen, creatinine, sodium, potassium, chloride, calcium, inorganic phosphorus, total protein, albumin and A/G ratio.

No treatment-related abnormalities were found during the gross necropsy examination nor were treatment-related microscopic changes noted in any of the organs or tissues examined. The investigators concluded that oral intake of 1,000 mg/kg-bw/day of *Bifidobacterium infantis* M63 powder ($7.6x10^{10}$ CFU *B. infantis/kg-bw/day)* for 91 days occurred without signs of toxicity. The NOAEL for this study was estimated to be above 1,000 mg/kg-bw/day *B. infantis* powder, the highest dose tested.

Table V-6. Summary of *in vitro* Studies with *B. longum* BB536			
Reference	**Objective**	**Study Design**	**Effect of *B. longum*[a,b]**
Araya-Kojima et al. 1995	Study the inhibitory effects of human-derived *B. longum* BB536 on harmful intestinal bacteria.	Co-cultivation with one of eight bacterial strains: *E. coli, Klebsiella pneumoniae, Clostridium clostridiiforme, C. perfringens, Bacteroids distasonis. B. fragilis, B. thetaiotaomicron* and *B. vulgatus*	-Inhibited growth of all bacteria strains. -Decreased the production of ammonia, but resulted in higher ammonia assimilation activity. -Decreased pH of cultures by producing lactic and acetic acids.
Grill et al. 1995c	Study the effects of 6 different bifidobacteria strains (including *B. longum* BB536) on two procarcinogens: nitrite and nitrosamines.	Cellular extracts: supernatant and membrane	-Growth of bifidobacteria not affected by nitrite concentrations below 50u mol l^{-1} but concentrations greater than 2000 u mol l^{-1} completely inhibited growth. -Acids produced by bacteria appeared to assist in nitrite elimination. -Nitrosamines had no effect on growth of bifidobacteria. -Only BB536 was able to metabolize nitrosamines by an intracellular mechanism.
Makras et al. 2006	Study the inhibitory activity of *B. longum* BB536 on Gram-negative pathogenic bacteria	Agar spot assay	-Strong antibacterial activity against *S. enterica* ser. Typhimurium SL 1344, *L. delbrueckii* subsp. *bulgaricus* LMG 6901 and E. *coli* C1845.
Tahri et al. 1995	Test the ability of *B. longum* BB536 and other bifidobacteria to uptake cholesterol and/or co-precipitate it with bile salts.	Growing and resting cell assays	-Resting cells did not interact with cholesterol -Growing cells resulted in 50% removal of cholesterol. -After several washings, researchers concluded that intense binding with cholesterol on the cell surface or uptake into the cell occurred. -Assimilation appeared to be dependent on cell growth and the presences of bile salts were not a prerequisite for significant cholesterol removal.

Table V-6. Summary of *in vitro* Studies with *B. longum* BB536			
Reference	**Objective**	**Study Design**	**Effect of *B. longum*[a,b]**
Takahashi et al. 2006	Study the effect of immunostimulatory oligodeoxynucleotide (ODN) BL07 isolated from the genomic DNA of *B. longum* BB536 on the ovalbumin-induced production of IgE and cytokine production in BALB/c mouse splenic cells.	Culture assays of ODN BL07	-Increased proliferation of B cells. -Increased Th1-type cytokine levels. -Inhibited IgE synthesis in dose-dependent manner. -Non-significantly increased in secretion of IgG2a.
a Results are significant unless noted otherwise. b Results are related to *Bifidobacterium longum* groups unless noted otherwise			

Table V-7. Summary of Animal Studies with *B.longum* BB536

Reference	Study Objective & Design	Animal Species and Number	Test Substance	Dose *Bifidobacterium longum*[a]	Treatment Duration	Results of *B. longum*[b,c]
Acute Studies - Rats						
Momose et al. 1979 (Japan) *Translated version used.*	Determine the LD_{50} of *B. longum* BB536 in mice.	20M/20F ICR mice	*B. longum* given orally and ip. 10^{12} CFU/g powder	Total Intake (oral): ~$5x10^{13}$ CFU/kg-bw 53.51 g/kg-bw (males) 54.99 g/kg-bw (females) Total Intake (ip): ~ $9x10^{11}$ CFU/kg-bw in high dose group 0.30, 0.39, 0.51, 0.66, and 0.86 g/kg- bw (ip.)	Oral: 3x in 24 hr ip: single exposure	**Oral** -No deaths in high dose group. -No abnormal conditions related to the general condition of the mice reported. Anatomical dissection of mice revealed nothing abnormal - No LD50 identified. **ip.** -LD_{50} reported to be 0.53 g/kg-bw ($5.3x10^{11}$ CFU per mouse) for male mice and 0.56 g/kg-bw ($5.6x10^{11}$ CFU per mouse) for female mice. -All mice died in high dose group; dissection revealed almost no remnant of the sample inside the abdominal cavity. -Abnormalities noted include raised hairs, reduced voluntary movements, unsteady walking, accelerated respirations, uneasiness, and closed eyes.
Repeat Dose Studies - Rats						
Challa et al. 1997	Study the effects of *B. longum* BB536 on induced colonic aberrant crypt foci (ACF) in rats.	29M F344 rats	*B. longum* or *B. longum* & lactulose	0.5% *B. longum* BB536 in diet $5.6x10^{9}$ CFU/kg-bw/d (given: $1.4 x10^{9}$ CFU/rat/d; assume mean bw of 0.25kg)	13 wk	-No effect on weight gain or food intake. -No change in diarrheal index, cecal pH, or cecal weight. -Decreased proximal, distal and total number of ACF. -Decrease in proximal and total number of aberrant crypts. -No effect on liver or colonic mucosa glutathione S-transferase levels.

Table V-7. Summary of Animal Studies with *B.longum* BB536

Reference	Study Objective & Design	Animal Species and Number	Test Substance	Dose *Bifidobacterium longum*[a]	Treatment Duration	Results of *B. longum*[b,c]
Igarashi et al. 1994 *Translated version used*	Investigate the effect of *B. longum* BB536 on whey calcium absorption and bone fracture properties in ovariectomized rats.	19F SD rats, 9-10 per group.	Whey calcium *B. longum* (group 2); or whey calcium, *B longum,* and lactulose (group 3)	$4.4x10^{10}$ CFU/kg (given: $10x10^{7}$ CFU/ml; 40ml per day milk; mean bw 0.09 k)	31 d	-Numerical increase in bifidobacteria on days 16 and 23 for group 2. -Increase in bifidobacteria in group 3. -Cecal pH lower and acetic and lactic acid concentrations higher in group 3 compared to control. -No effect on serum Ca, P, creatinine, or total protein levels. -Increase in breaking energy, breaking force, area and height of femur in group 3. -No effect on body weight among groups. -Macroscopic examination revealed slight enlargement of cecum in group 3.
Kulkarni and Reddy 1994	Evaluate the of *B. longum* BB536 on preneoplastic lesions in the colon and on fecal bacterial (3-glucuronidase activity in rats	11M F344 rats	2 dosages of *B. Longum* BB536	1.5% or 3% of diet $1.8x10^{10}$ CFU/kg-bw/d or $3.6x10^{10}$ CFU/kg-bw/d (given: $2x10^{10}$ CFU/g culture medium; assume default daily intake of 15 g and bw of 0.25 kg)	<12wk	-Decrease in formation of ACF induced by azoxymethane (AOM) in low- and high-dose treatment groups. -Decrease in crypt multiplicity in both treatment groups. -No effect on body weight. -Decrease in cecal (3-glucuronidase activity.

Table V-7. Summary of Animal Studies with *B.longum* BB536

Reference	Study Objective & Design	Animal Species and Number	Test Substance	Dose *Bifidobacterium longum*[a]	Treatment Duration	Results of *B. longum*[b,c]
Reddy and Rivenson 1993	Evaluate the effect of *B. longum* BB536 on 2-amino-3-methyylimidazo[4,5-f]quinoline (IQ) in rats.	30M/30F F344 rats IQ group; 9M/9F F344 rats non-IQ group	*B. longum* BB536 diet (with or without IQ).	0.5% *B. longum* diet $6x10^9$ CFU/kg-bw/d (given: $2x10^{10}$ CFU/g culture medium; assume default daily intake of 15 g diet and bw of 0.25 kg)	58 wk	-Inhibited IQ-induced incidence of colon and liver tumors and multiplicity of colon, liver and small intestine tumors in males. -Inhibited mammary tumor multiplicity in females. -No effect on body weights throughout study.
Singh et al. 1997	Investigate colon tumor inhibitory activity of *B. longum* BB536 in rats.	42M F344 rats AOM group; 12 F344 rats Saline group	AOM treated *B. longum* diet or saline treated *B. longum* diet.	2% *B. longum diet* $2.4x10^{12}$ CFU/kg-bw/d (given: $4x10^{10}$ CFU/g diet; assume default daily intake of 15 g diet and bw of 0.25 kg)	43 w k	-No effect of body weight. -Decreased incidence of colon adenocarcinomas. -No effect on incidence of small intestine tumors. -Decreased AOM induced colonic mucosal cell proliferation -Decreased expression of ras-p21 oncoprotein in AOM treated animals.

Table V-7. Summary of Animal Studies with *B.longum* BB536

Reference	Study Objective & Design	Animal Species and Number	Test Substance	Dose *Bifidobacterium longum*[a]	Treatment Duration	Results of *B. longum*[b,c]
Single Dose Studies - Rats						
Watabe 2004	Evaluate the potential of *B. longum* BB536 to induce DNA damage in rats given 2-acetylaminofluorene (2-AAF) or dimethylnitrosamine (DMN).	12M SD rats	*B. longum* via oral gavage	500 or 2000 mg/kg- bw (10^{10} or 10^{14} CFU, respectively.)	Single dose Sampled at 2 or 16 hr	-No effect on body weight. -Treatment did not induce DNA damage in hepatocytes isolated from treated rats.
Single Dose Studies - Mice						
Namba et al. 2003	Study the effects of *B. longum* BB536 on *E. coli* in mice.	16BALB/c M/F mice GF or MA	*B. longum y* given orally 29 days prior to infection withE. *coli*	$1x10^7$ CFU	Single dose	-All mice in treatment groups survived up to end of infection, all control GF mice died. -*E. Coli* counts in *B. longum* group were inhibited to 10% of the counts in the GF mice. -No reports of diarrhea in any of the groups. -Some mice in the treatment group showed "mild lesions in the kidney."
Sekine et al. 1994	Investigate the immune-related effects of *B. longum* BB536 in mice.	BALB/c M mice (n not reported)	*B. longum* injected intraperitone ally (ip)	25 mg $5.7x10^5$ CFU	Single dose Observed for 3 hr	-*B. longum* induced the expression of IL-1β, IL-6, IL-10, and TNF-a mRNA in mouse peritoneal cells.

Table V-7. Summary of Animal Studies with *B.longum* BB536

Reference	Study Objective & Design	Animal Species and Number	Test Substance	Dose *Bifidobacterium longum*[a]	Treatment Duration	Results of *B. longum*[b,c]
	Evaluate the antitumor activity of *B. longum* BB536 against TNF-∝-sensitive Meth A tumors. tumor cells ($1x10^5$).	20M Balb/c mice	*B. longum* administered subcutaneous sly.	1 mg or 5 mg CFU/mouse not given.	Single dose Observed for 14 d	-Tumor incidence was suppressed in low- and high-dose treatment groups.
Yamazaki et al. 1982	Investigate the resistance of *Bifidobacterium longum* inoculated mice to the lethal activity of *Escherichia coli,* strain O111:K58 (10^{10} CFU/mouse given orally)	16M/F mice Germ free (GF) or Gf-BALB-nu	Heat-killed *B. longum* intragastric inoculation 3 weeks prior to exposure.	$2x10^{10}$ CFU (given: $5x10^{10}$ cell/ml with 0.4ml administration)	Single dose observed for 14 d	-*E. coli* did not kill any treatment group mice 18 hr after dosing, 7 out of 11 (64%) GF mice died. -Non-significant difference in mortality rate: 62.5% (treatment) vs. 75% (control). -All mice had large numbers *of E. coli* in their ceca. -Viable *E. coli* in organs (liver, spleen, kidney, and lung) of inoculated mice disappeared by day 7, but were persistent in GF mice through the end of the study.
Yamazaki et al. 1985	Investigate the resistance of *B. longum* inoculated mice to the lethal activity of *Escherichia coli* strain O111:K58. ($4x10^8$ to $5x10^8$ CFU/mouse)	90 mice GB or GF	Intragastric inoculation with *B. longum* 2 to 3 weeks prior to exposure.	$1x10^7$ CFU (given: $2.5x10^7$ cell/ml w/ 0.4ml administration)	Single dose Observed for 18 hr	-Diet containing auto-claved *B. longum* had no effect *on E. coli* challenge. -At 2 weeks post *B. longum* inoculation, all mice died after exposure to *E. coli.* -At 3 weeks post-B. *longum* inoculation, fewer than half of mice died after *E. coli* exposure. -Decreased viable *E. coli* and endotoxin in liver, spleen, kidney, and blood of inoculated mice compared to GF mice. -Inoculated mice also showed decreased susceptibility to the lethal effects of endotoxin.

Table V-7. Summary of Animal Studies with *B.longum* BB536

Reference	Study Objective & Design	Animal Species and Number	Test Substance	Dose *Bifidobacterium longum*[a]	Treatment Duration	Results of *B. longum*[b,c]
Repeat Dose - Mice						
Tomoda et al. 1981 *Translated version used.*	Study of the effect of bifidus milk on intestinal flora in immunocompromised mice. Parallel design. No statistical analysis.	28 C3H/He mice undergoing chemotherapy with three different agents (prenisoline, 5-Fu or cyclophophamide)	Milk containing *B. longum* and L. *acidophilus*	10^8 CFU, 1x daily. $4x10^9$ CFU/kg-bw/d (assume default bw of 0.025 kg)	3 mo	-Numbers of *Klebsiella, Citrobacter,* other Gram-negative rod bacteria, *E. coli,* and *Candida* were lower in the treatment groups than in the control groups for all chemotherapy agents.
Tomoda et al. 1986 *Translated version used.*	Study of the colonization and proliferation of bifidobacteria in mice.	*5* C3H/He mice	Milk containing *B. longum* and L. *acidophilus*	10^8 CFU, 1x daily. $4x10^9$ CFU/kg-bw/d (assume default bw of 0.025 kg)	6 mo	-*B. pseudolongum* was above baseline levels at 2, 4, and 6 mo of treatment and 1 month post-treatment. -No *B. longum* was seen at baseline; levels were 10^{10} - 10^{11} CFU during feeding and returned to zero at 1 mo post-treatment.

a Standardized default animal feed intakes and body weights were used to calculate CFU/kg-bw/d intakes of *B. longum* for repeat-dose studies when mean animal body weights and diet intakes were not directly stated in the studies,
b Results are significant unless noted otherwise.
c Results are related to *Bifidobacterium longum* groups unless noted otherwise.

Table V-8. Studies of *B. longum* BB536 Ingestion by Healthy Adults

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose B. *longum*	Treatment Duration	Results of *B. longum* Ingestion[a,b]
Ballongue et al. 1993 (France) *Translated version used.*	Study effects of fermented milk containing *B. longum* BB536 on fecal microflora in adults. *(B. animalis* also tested.) Parallel study Statistical analysis not performed	n=9	Fermented milk containing *B. longum* BB536	≥1.25xl0^9 CFU, 3x per day Total Daily Dose: ≥3.75xl0^9 CFU	3 wk	-Numerical increase in total anaerobes and bifidobacteria after 3 wk treatment period compared to baseline values and control group. -Numerical decrease in fecal concentrations of clostridia, bacteroides, and *E. coli* after 3 wk treatment period compared to baseline values and control group. -Bifidobacteria concentrations were lower at 3 wk post-treatment, but remained higher than baseline levels. -Fecal anaerobes, clostridia, bacteroides and *E. coli* increased at 3 wk post- treatment, but remained below baseline levels.
	Study effects of fermented milks with *B. longum* BB536 on fecal microflora in adults. (Other bifidobacteria tested included: *B. longum* BB536S15, ATCC 15707 and ATCC 15707S42) Parallel study Statistical analysis not performed	n=18	Fermented milk containing *B. longum* BB536 or BB536-S15 (labeled strain)	≥1.25xl09 CFU, 3x per day Total Daily Dose: ≥3.75xl0^9 CFU.	3 wk	-Numerical increase in total anaerobes and *Bifidobacteria* at end of treatment period. -Numerical increase in *Bifidobacterium* at 3 weeks post-feeding period. -Decrease in clostridia at end of treatment period. -Decrease in coliforms and bacteroides end of treatment period and at 3 weeks post-treatment period. - BB536S15 80% of bifidus fecal flora at end of treatment and 60% at 3 weeks post-feeding.

Table V-8. Studies of *B. longum* BB536 Ingestion by Healthy Adults

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose B. *longum*	Treatment Duration	Results of *B. longum* Ingestion[a,b]
Ebisawa et al. 1985 (Japan) *Translated version used.*	Study the effects of bifidobacteria on gastrointestinal conditions and defecation in hospitalized pregnant women. Questionnaire study Statistical analysis not	n=110; (y=20s & 30s)	Yogurt containing *Bifidobacterium* and lactulose.* *Presence of strain BB536 confirmed by Morinaga.	$>10^{10}$ CFU, 1x daily	6 d	-25% reported improved GI conditions. -Frequencies of breaking wind, rumbling bowels, and gas in the bowels decreased throughout feeding period. -Frequencies of constipation and diarrhea decreased during the feeding period. -Prevalence of those defecating < 1/d decreased during the feeding period.
Nanba et al. 2006 [Abstract only] *Translated version used.*	Study effect of *B. longum* BB536 on rates of infections including influenza during wintertime in elderly adults in a nursing home administered the influenza vaccine. Randomized, double-blind, placebo controlled	N=unknown; >65y	Sachets of *B. longum* BB536 powder	10^{11} CFU/d	14 wk	-Decrease in number of subjects contracting influenza, developing fevers and using antibiotics. -Higher bactericidal activity of neutrophils and NK cell activity at wk 5. -No effect on levels of antibody titers

Table V-8. Studies of *B. longum* BB536 Ingestion by Healthy Adults

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose B. *longum*	Treatment Duration	Results of *B. longum* Ingestion[a,b]
Ogata et al. 1997 (Japan)	Study the effects of *B. longum* BB536 on the fecal microflora, putrefactive substances, enzyme activities and organic acids in the feces of adults. Crossover study.	n=12, 21-57 y	Milk containing *B. longum* BB536 at two different dosages.	2×10^9 CFU/d or 2×10^{10} CFU/d	1 wk	-Decrease in ammonia content during treatment period for high-dose group. -Increase in fecal water percent in high dose group. -No changes in fecal microflora count or relative percentages.
	Study the effects of *B. longum* BB536 on defecation frequency and characteristics in constipated individuals. Crossover study.	n=40F, 20-28 y	Milk containing *B. longum* BB536	$2x10^9$ CFU; 1x per day	3 wk	-Increase in defecation frequency and stool softness in treatment group compared to control group.
Ogata et al. 1999 (Japan)	Study effects of yogurt supplemented with *B. longum* BB536 on intestinal microflora counts and activity in healthy adults. Crossover study	n=6; 2M/4F, 21-42 y	Yogurt with *B. longum* BB536* * also contained: *Streptococcus thermophilus* and *L. delbrueckii* subsp.*bulgaricus*	$>5x10^9$ CFU, 1x daily	2 wk	-Increase in fecal *Lactobacillus* count. -Increase in proportion of *Bifidobacterium.* -Increase in SCFA and VFA concentrations. -Lower fecal ammonia levels during treatment as compared to post-treatment.
Seki et al. 1978 (Japan) *Translated version used.*	Study the effects of milk containing *B. longum* and *L. acidophilus* on regulatrity in elderly adults with constipation. Crossover study.	n=18; 3M/15F, >60 y	Milk containing *B. longum* BB536 milk* * also contained *L. acidophilus* and *S. thermophilus*	2×10^{10} CFU, 1x per day	10 d	-Fecal samples were analysed for *B. longum* content in 5 of the subjects. -Increase in stool frequency.

Table V-8. Studies of *B. longum* BB536 Ingestion by Healthy Adults

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose B. *longum*	Treatment Duration	Results of *B. longum* Ingestion[a,b]
Tomoda et al. 1990 & 1991 (Japan) *Translated version used.*	Study the effect of bifidobacteria on GI function in adults. Crossover study.	n=10; 20-60 y	1 treatment: Yougurt containing *B. longum* BB536* * also contained: *Streptococcus thermophillus* and *L. delbrueckii* subsp. *bulgaricus*	>$1.3x10^{10}$ CFU/d	6 wk	-Administered *B. longum* samples taken from feces of 3 of the 10 subjects. -Decrease in ammonia levels in all 3 tested subjects. -Increase in fecal *Bifidobacterium.* -No changes in blood chemistry or liver function. -Participants reported no discomfort from the treatment nor were any reports of constipation or diarrhea made in the evaluation forms.
	Study the effect of yogurt containing *B. longum* with or without lactulose on GI function in adults. Crossover study.	n=10, 5F/5M; 20-60 y.	4 treatments: Yogurt containing *B. longum* BB536 and/or lactulose or Control yogurt (plain)	>$1.3x10^{10}$	3-6 wk	-Increase in bifidobacteria population and decrease in ammonia. -Bifidobacteria returned to pre-treatment levels when tested at end of 3-mos interval. -No observed changes in blood chemistry. -No changes in appetite or body weight reported. -No side effects reported by participants in study evaluation forms.
Xiao et al. 2006a (Japan)	Study effect of *B. longum* BB536 administered in a yogurt matrix on relieving Japanese cedar pollinosis in otherwise healthy adults with >2-year clinical history of JPCsis. Randomized, double-blind, placebo-controlled.	N=20; mean 37 y	Yogurt containing BB536* *Also contained: *S. thermophilus* and *L. delbrueckii* subspl. *bulgaricus*.	>$4x10^{9}$ CFU/d (Assuming $2x10^{7}$ CUF/g yogurt and 200g yogurt ingestion per day)	14 wk	-Decrease in self-reported eye symptoms between weeks 5 and 14. -No changes in nasal itching, rhinorrhea, nasal blockage, sneezing or throat symptoms. -Fewer moderate to severe subjective symptoms reported in treatment group compared to control. -Higher levels of IFN-γ compared to control group at 5, 9 and 14wk. -No changes in total IgE, JCP-specific IgE, IL-10 levels compared to control. -Higher eosinophil rate in control group at wk 9 and 14. -All participants completed study. -Compliance rate of >95% for all participants

Table V-8. Studies of *B. longum* BB536 Ingestion by Healthy Adults

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose B. *longum*	Treatment Duration	Results of *B. longum* Ingestion[a,b]
Xiao et al. 2006b (Japan)	Study effect of administration of powder containing *B. longum* BB536 in a milk matrix on relieving Japanese cedar pollinosis in otherwise healthy adults with >2-year clinical history of JPCsis. Randomized, double-blind, placebo-controlled.	n=22; mean 36.5 y	Milk containing *B. longum* BB536	10^{11} CFU/d, Administered as 2 doses daily.	13 wk	-Reduction in number of subjects prematurely terminating study due to severe symptoms. -Decrease in subjective symptoms of rhinorrhea, nasal blockage, nasal itching, sneezing, throat symptoms and composite scores. -Decrease in total IgE levels at 4, 8 and 13 wk compared to control. -No participants left the trial prematurely due to adverse effects.
Xiao et al. 2007 (Japan)	Study effect of *B. longum* BB536 on relieving Japanese cedar pollinosis in otherwise healthy adults with >2- year clinical history of JPCsis placed an environmental exposure unit (EEU) for 4 hr post-treatment period. Double-blind; Crossover study.	n = 24; mean 37.6 y	Milk containing freeze-dried *B. longum* BB536	10^{11} CFU/d, Administered as 2 doses daily	4 wk	-Decrease in eye symptoms at 30 min in EEU. -No difference between treatment group and control for other subjective symptoms. -Decrease in total number of days of oral medication and eye drop usage post-EEU between treatment and placebo group. -Not changes in total or JCP-specific IgE levels were observed between groups. -One drop-out due to failure to undergo second pollen exposure. -Two other subjects excluded from final evaluation due to development of colds at time of second exposure test
Yaeshima et al. 1997 (Japan)	Study the effects of probiotic cocktail on intestinal environment in healthy adults. Crossover study.	n=11F	Yogurt containing *B. longum* BB536* *Also contained: *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*	2×10^9 CFU/d	2 wk	-Increase in number and relative percentage of *Bifidobacterium* compared to control. -No changes in fecal pH, ammonia, % H_2O or SCFA

Table V-8. Studies of *B. longum* BB536 Ingestion by Healthy Adults

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose B. *longum*	Treatment Duration	Results of *B. longum* Ingestion[a,b]
	Study the effects of yogurt containing *B. longum* on fecal characteristics and defecation frequency in healthy adults. Crossover study.	=39F	2 treatments Yogurt containing *B. longum* BB536* or Control (standard yogurt). *(same as above)	2 x 10^9 CFU/d	3 wk	-Increase in defecation frequency. -Feces were softer and yellow in color.
Yaeshima et al. 1998 (Japan)	Study the effects of bifidobacteria on fecal characteristics and defecation frequency in healthy adults. Crossover study.	N=41F; mean 42 y	Yogurt containing *B. longum* BB536* *Also contained: *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*	>2 x 10^9 CFU/d	2 wk	-Increase in defecation frequency. -Researchers noted color change to yellow.
Yaeshima et al. 2001 (Japan) *Translated version used.*	Study the effect of non-fermented BB536 milk on defecation frequency and characteristics in healthy adults with constipation. Crossover study	n=43 F; mean 43 y	Non-fermented milk containing *B. longum* BB536* *Also contained: *Lactobacillus gasseri* and 376mg/bottle Ca++	>2 x 10^9 CFU; 180 ml milk per day	2 wk	-First 2 days of treatment not included in analysis. -Increase in defecation frequency. -Number of days with defecation increased. -No adverse events or changes in physical condition reported in questionnaires.

a Results are significant unless noted otherwise.
b Results are related to *Bifidobacterium longum* groups unless noted otherwise.

Table V-9. Studies of *B. longum* BB536 Ingestion by Compromised Children and Adults

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose B. *longum*	Treatment Duration	Results of *B. longum* Ingestion[a,b]
De Vrese et al. 2001 (Germany) [Poster presentation]	Study the effects of *B. longum* BB536 on GI and microflora in patients with *H. pylori* infection taking antibiotics for one week during feeding. Controlled study.	n=34	Yogurt containing *B. longum* BB536	$1.25x10^{10}$ CFU; 2x daily Total Daily Dose: $2.5x10^{10}$ CFU.	8 wk	-Decreased incidence of diarrhea. -Reduced incidence of diarrhea during antibiotic therapy. -Improved GI symptoms after antibiotic therapy. -No change in fecal microflora.
Inoue et al. 2006 [Abstract only] *Translated version used.*	Study effect of *B. longum* BB536 on ulcerative colitis in adult patients in conjunction with conventional treatments.	n=8	*B. longum* BB536	2 to $3x10^{11}$ CFU/d	12 wk	-Non-significant trend in the decrease of CIA during treatment compared to before. -Two patients discontinued 5-ASA treatment. -Investigators reported increases in MDR-1, COX-2 and TFGβ expression after *in vitro* addition of BB536 to Caco-2 cells. -Researchers concluded that treatment was useful in the treatment of mild to moderate cases of UC.
Kageyama et al. 1984 (Japan)	Study the effects of *B. longum* and *L. acidophilus* in milk on fecal microflora in leukemia patients during and after chemotherapy. Nonrandomized, controlled observational study. No statistics performed	n=28	Milk containing *B. longum* BB536* *Also contained: *L. acidophilus*	$2 x10^{9}$ CFU; 1x daily	2-3 mo	-Samples taken from 16 of the 28 treatment subjects. -Decrease in patient fecal *Proteus vulgaris* and *Pseudomonas.* -Decrease in fecal *Candida.* - Decrease in number of patients testing positive for urine indicant and blood endotoxin. -Increased fecal count of bifidobacteria and decreased counts of *E. coli*

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Table V-9. Studies of *B. longum* BB536 Ingestion by Compromised Children and Adults

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose B. *longum*	Treatment Duration	Results of *B. longum* Ingestion[a,b]
Kageyama et al. 1987 (Japan) *Translated version used*	Study the effects of probiotic cocktail on fecal microflora in leukemia patients who hadn't used antibiotics. Nonrandomized clinical observational study.	n=3	Milk containing *B. longum* BB536* (n=1) and/or *B. longum* BB536 powder mixed with lactulose (n=2) *Milk (not powder) also contained: *L. acidophilus*	10^9-10^{10} CFU/d	6 mo	-Increases in *Bifidobacterium* and *L. acidophilus* in all patients.
Sekine et al. 1985 (Japan) *Translated version used.*	Study the effects of *Bifidobacterium* on peripheral leukocytes and red blood cells in children with cancer undergoing chemotherapy. Observational study.	n=8; 4-12 y	Milk containing *B. longum* BB536 and vitamins	Ages 4-6: $7x10^8$ CFU Ages 7-12: 10^9 CFU	1 yr	-Decrease in RBC mean corpuscular volume (MCV) in 4 of the 7 treatment subjects tested. -Enhanced chemiluminescent reaction of circulating phagocytes.
Tomoda et al. 1981 (Japan) *Translated version used.*	Study of the effect of milk containing *B. longum* and *L. acidophilus* on intestinal flora in cancer patients given anticancer or immunosuppressive drugs. Controlled study. No statistical analysis.	n=28 (10 healthy controls also observed)	Milk containing *B. longum* BB536* *also contained: *L. acidophilus.*	$2x10^9$ CFU; 1x per day	2-6 mo	-Suppressed proliferation of *Pseudomonas, P. vulgaris* and Candida

Table V-9. Studies of *B. longum* BB536 Ingestion by Compromised Children and Adults

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose B. *longum*	Treatment Duration	Results of *B. longum* Ingestion[a,b]
Tomoda et al. 1986 (Japan) *Translated version used*	Study of the colonization and proliferation of bifidobacteria in patients with chronic hematological diseases not using antibiotics. Observational study	n=3	Milk containing *B. longum* BB536* *also contained: *L. acidophilus.*	$2x10^9$ CFU; 1x per day	3-, 6-, and 9-mo	-*B. longum* detected in all feces during treatment. -*B. longum* detected in feces 1 month after feeding in 1 out of 3 subjects
Tomoda et al 1988 (Japan)	Study the effect of milk containing *B. longum* and *L. acidophilus* on intestinal *Candida* overgrowth and infection in leukemia patients undergoing chemotherapy. Controlled study.	n=28 (34 reference group subjects)	Milk containing *B. longum* BB536* *Also contained: *L. acidophilus*	$2x10^9$ CFU/d	>3 mo	-Candida fecal count was lowered in half of the study population; unclear if patients consumed *B. longum* or *B. infantis.*

a Results are significant unless noted otherwise.
b Results are related to *Bifidobacterium longum* groups unless noted otherwise.

Table V-10. Studies of *B. longum* BB536 Ingestion by Infants

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose *B. longum*	Treatment Duration	Results of *B. longum* Ingestion[a,b]
Akiyama et al. 1994 (Japan) *Translated version used.*	Study the effects of *B. longum* on the intestinal microflora of preterm infants. Randomized controlled trial. No statistical analysis appears to have been performed.	n=5	0.5 g of *B. longum* BB536 dissolved in 1 ml water and administered intragastrically.	5×10^8 CFU; 1x per day	8 wk	-Infants in treatment group responded differently to supplementation.
Bennet et al. 1992 (Sweden)	Study of the colonization of the gut of fullterm infants by oral probiotic treatments including *B. longum* just after antibiotic treatment. 2 individual treatments of *B.* breve BB576 and L. *acidophilus* LAC343 also administered. No statistics performed.	n=11; 0-8 wk (3 subjects in non-antibiotic reference group)	Powdered capsules containing *B. longum* BB536 or Cocktail capsule including *B. longum* BB536	3×10^9 CFU; 3x per day with meals Total Daily Intake: 9×10^9 CFU	5 d	-All infants acquired anaerobic bacteria by day 5. - *B. longum* isolated least frequently from feces.

a Results are significant unless noted otherwise.
b Results are related to *Bifidobacterium longum* groups unless noted otherwise.

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Table V-11. Summary of Animal Studies with other Strains *of Bifidobacterium longum*						
Reference	**Study Objective & Design**	**Animal Species and Number**	**Test Substance**	**Dose *B. longum***	**Treatment Duration**	**Results of *B. longum* Administration[a,b]**
Choi et al. 2005	Investigate the safety of *B. longum* SPM1205 orally administered to rats.	6M Sprague-Dawley rats	*B. longum* SPM1205 extracted from healthy adult Korean volunteers	$1x10^9$ CFU/kg-bw/d	4 wk	-No clinically abnormal signs observed. -No differences in body weight or growth rates compared to control. -No changes in blood biochemistry. -No histopathological abnormalities observed at necropsy. -100-fold increase *in Bifidobacterium* spp. in fecal samples. -Inhibition of harmful enzymes observed in fecal assays.
Hidemura et al. 2003	Study effects of orally administered *B. longum* OLL6001 in mice with diet restriction-induced immunosuppression in a glycogen-induced peritonitis model.	20M ICR mice (another 20 in control group)	Standard mouse chow with 1% B. *longum* OLL6001	0.4 g/d ($1x10^4$ CFU/ml)	7 d	-No difference in body weight change between the control and treatment groups at end of 7 day. -PEC and neutrophils were higher in treatment group 4 hr after glycogen injection. -Cytokine concentrations of IL-6 and IL-10 were higher in treatment group compared to control at 2 hr post-glycogen injection. -CD 18 and DC62L expression on circulating PMNs were higher in treatment group compared to control at 4 hr post-glycogen injection.

a Results are significant unless noted otherwise.
b Results are related to *Bifidobacterium longum* groups unless noted otherwise.

Table V-12. Summary of Healthy Adult Studies with Other Strains *of Bifidobacterium longum*

Reference	Study Objective & Design	Treatment Population	Test Substance	Dose B. *longum*	Treatment Duration	Results of *B. longum* Ingestion[a],[b]
Makelainen et al. 2003 (Finland) Double-blind, controlled study; parallel design	Assess the safety of *B. longum* 2C and 46 in healthy human subjects.	n=19; 19-60 y	*B. longum* 2C and *B. longum* 46	$4x10^9$ CFU/d (strains combined); 1 capsule 2x daily	3 wk	-No changes in immune response (measured by phagocytic activity) was observed. -No differences in intestinal complaints were reported between groups. -No negative clinical effects were observed in any of the participants.

a Results are significant unless noted otherwise.
b Results are related to *Bifidobacterium longum* groups unless noted otherwise.

Table V-13. Summary of Acute and Subchronic Toxicity Animal Studies with Other Species of Bifidobacteria

Reference	Study Objective & Design	Animal Species and Number	Test Substance	Dose Bifidobacteria	Treatment Duration	Results of Bifidobacteria Administration[a,b]
Bifidobacterium breve						
Onishi 1992 [unpublished]	Invest acute toxicity of *B. breve* M- 16 V powder orally administered to rats.	20M/20F Sprague-Dawley rats	*B. breve* M-16 V powder at two different doses Source: Morinaga Milk Inc.	$3.0x10^{11}$ or $6.0x10^{11}$ CFU/kg-bw (Assuming viable bacteria count of at least $1x10^{11}$ per g) 3,000 or 6,000 mg/kg-bw powder	Single dose	-Clinical conditions and behavior not affected by test substance. -During days 8 through 10 of observation after administration of test substance, male body weight decreased in high-dose group; difference disappeared by day 12. -Necropsy revealed no abnormalities. -No deaths related to test substance observed. -Researchers concluded $6.0x10^{1}1$. CFU/kg-bw to be non-toxic.
Ikeya 2005b [unpublished]	Investigate the safety of *B. breve* M- 16 V powder orally administered to rats.	10M10F Sprague-Dawley rats	*B. breve* M-16 V powder Source: Morinaga Milk Inc.	$2.3x10^{11}$ CFU/kg-bw/d 1,000 mg/kg-bw/d powder	91 d	-Clinical conditions and behavior not affected by test substance. -No changes in body weight or food consumption observed compared to control. -No changes in animal organ weights. -No treatment-related abnormalities found in urinalysis, ophthalmoscopic, hematological, blood chemistry or histopatholicogal examinations. -Researchers noted increase in MCH and bilirubin in male treatment-group; changes were not considered to be related to treatment. -Researchers determined a NOAEL of >1,000 mg/kg-bw/d powder ($2.3x10^{11}$ CFU/kg-bw/d *B. breve),* the highest dose tested.

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Table V-13. Summary of Acute and Subchronic Toxicity Animal Studies with Other Species of Bifidobacteria

Reference	Study Objective & Design	Animal Species and Number	Test Substance	Dose Bifidobacteria	Treatment Duration	Results of Bifidobacteria Administration[a,b]
Bifidobacterium infantis						
Onishi 2001 [unpublished]	Invest acute toxicity of *B. infantis* M-63 powder orally administered to rats	5M/5F Sprague-Dawley rats	*B. infantis* M-63 Powder Source: Morinaga Milk Inc.	$3.2x10^{11}$ CFU/kg-bw (Assuming viable bacteria count of at least $8x10^{10}$ per g) 4,000 mg/kg-bw powder	Single administration	-Clinical conditions and behavior not affected by test substance. -Body weights increased at normal rates for all groups. -Necropsy revealed no abnormalities. -Researchers determined LDLo to be greater than $3.2x10^{1}1$ CFU/kg-bw.
Ikeya 2005a [unpublished]	Investigate the safety of *B. infantis* M-63 powder orally administered to rats.	10M/10F Sprague-Dawley rats	*B. infantis* M-63 powder Source: Morinaga Milk Inc.	$7.6x10^{10}$ CFU/kg-bw/d 1,000 mg/kg-bw/d powder	91 d	-Clinical conditions and behavior not affected by test substance. -No changes in body weight or food consumption observed compared to control group. -No treatment-related abnormalities found in urinalysis, ophthalmoscopic, hematological, blood chemistry or histopatholicogal examinations. -Researchers noted decrease in absolute and relative weights of seminal vesicles in males and relative weights of spleen in females; changes were not considered to be related to treatment. -Researchers determined a NOAEL of >1,000 mg/kg-bw/d powder ($7.6x10^{10}$ CFU/kg-bw/d *B. infantis),* the highest dose tested.

a Results are significant unless noted otherwise.
b Results are related to *Bifidobacterium longum* groups unless noted otherwise.

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SPHERIX

THE GENERALLY RECOGNIZED AS SAFE STATUS OF *BIFIDOBACTERIUM LONGUM* BB536 IN FOODS

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of food and food ingredients, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized as Safe (GRAS) status of the intended uses of *Bifidobacterium longum* BB536. The information for *B. longum* BB536 is summarized in the GRAS determination document, Generally Recognized As Safe Determination for the Use of *B. longum* BB536 in Selected Foods.

B. longum BB536 is intended for use as a dietary ingredient at a maximum concentration at end of shelf life of 1×10^{10} CFU per serving in breads/baked goods, cereals, dairy products/dairy-based foods and dairy substitutes, fruit products, and selected miscellaneous foods. Loss of *B. longum* BB536 during storage of representative foods requires that 1 to 10 times the target concentration of 1×10^{10} CFU per serving must be added at manufacture to meet the target concentration at the end of shelf life. The intended uses of *B. longum* BB536 manufactured consistent with current Good Manufacturing Practice and meeting the specifications described in the GRAS determination document are safe and Generally Recognized As Safe (GRAS) based upon scientific procedures as described under 21 CFR §170.30(b), and corroborated by a history of safe exposure and evidence of safety of other bifidobacterial strains. The determination of GRAS is based on the following:

- *B. longum* BB536 is a Gram-positive anaerobic bacterium. *B. longum* BB536 was originally isolated from a healthy infant in 1969. The bacterium has been deposited with the American Type Culture Collection (ATCC) and is designated BAA-999TM. The maintenance of the original frozen culture has been tightly controlled to ensure purity and stability of the strain.

- Product specifications are set to assure that *B. longum* BB536 is suitable for use in food.

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- *B. longum* BB536 has been tested for parameters outlined in the Food and Agriculture Organization of the United Nations/World Health Organization's (FAO/WHO) guidelines for the evaluation for microbes for probiotic use in foods. Results from these tests provide evidence that *B. longum* BB536 is safe for use in foods, namely:

  - Appropriate determination of antibiotic resistance parameters has been established. International efforts to develop standardized approaches for assessment of antibiotic resistance of microbes destined for food use are in progress. The concern is that non-pathogeneic, non-toxigenic microbes might serve as a source of antibiotic resistance genes that might be transferred *in vivo* to less innocuous members of the intestinal microbiota. The most comprehensive approach to assessing antibiotic resistance is to (1) test for phenotypic resistance, and (2) using known antibiotic resistance gene sequences, either probe total bacterial DNA for the presence of these genes or conduct genomic sequencing and look for the relevant gene sequences. In the absence of such genetic information (as is the case with *B. longum* BB536) confirming the absence of antibiotic resistance genes, the phenotypic analysis is of increased importance. The strategy recommended by the European Union ACE ART project is that phenotypic resistance for multiple strains of one species is established and the strain in question is compared to the norm for the species to determine if there are antibiotic resistances expressed outside this norm. Data on antibiotic susceptibility (testing by PROSAFE) established minimum inhibitory concentration (MIC) levels for *B. longum* BB536 and compared these to cut-off levels for multiple strains of *B. longum* on the basis of MIC distribution for 15 antibiotics. Results determined that no acquired resistances were detected for *B. longum* BB536 based on MIC comparison. Additional testing of homology between known antibiotic resistance genes in lactic acid bacteria and the *B. longum* BB536 genes showed that the *B. longum* BB536 genes do not include these antibiotic resistance genes. Results from testing of *B. longum* BB536 also indicate that the strain is sensitive to tetracycline and does not contain a tetracycline resistant gene. The available

antibiotic resistance pattern suggests that *B. longum* BB536 does not present concerns for antibiotic resistance in humans.

- *B. longum* BB536 produces predominantly L-lactic acid, while production of D-lactic acid is negligible.

- *B. longum* BB536 has been reported to deconjugate bile salts. The production of deconjugated bile salts was concurrent with bacterial growth, and deconjugated bile salts were the only compounds produced.

- Results from comparisons of amino acid sequences of known bacterial toxins with sequences of the predicted proteins from the genomic sequence of *B. longum* BB536 and genomic sequences of three known pathogens with sequences of the predicted proteins from the genomic sequence of *B. longum* BB536 indicate that there is no significant homology.

- *B. longum* BB536 was not observed to have hemolytic activity.

- Results from repeat dose studies of up to 43 weeks of *B. longum* BB536 administered to rats show no treatment effects on body weight, body weight gain, or feed intake at doses up to 2×10^{12} CFU/kg bw/day. The studies provide support for the safe use of *B. longum* BB536.

- Clinical studies involving the administration of *B. longum* BB536 to healthy and unhealthy adults, children and infants at doses up to 10^{11} CFU per day provide support for the safe and well-tolerated use of *B. longum* BB536.

- Research on bifidobacteria has been conducted on several strains of *B. longum*; results from studies of other strains of *B. longum* provide corroborative evidence for the safety of human consumption of *B. longum* BB536.

- Assuming addition of 1×10^{10} CFU of *B. longum* BB536 per serving in the target food categories, the estimated mean and 90th percentile 2-day average intakes of *B.*

longum BB536 from all proposed use categories combined in the population ages 2 years and older are 7.5×10^{10} and 1.2×10^{11} CFU, respectively.

Therefore, based on our independent and collective critical evaluation of the available information on *B. longum* BB536 and other bifidobacterial strains, we, members of the Expert Panel, conclude that the intended uses of *B. longum* BB536, produced in accordance with current Good Manufacturing Practice and meeting the specifications referenced in the GRAS determination document, are safe and are Generally Recognized as Safe (GRAS) based on scientific procedures and corroborated by history of safe exposure. Because the intended uses of *B. longum* BB536 by Morinaga Milk Industry Co. Ltd., are safe and GRAS, it is excluded from the definition of a food additive, and thus may be marketed for these uses without the need to promulgate a specific food additive regulation under 21 CFR.

It is our opinion that other experts qualified by training and/or experience to evaluate the safety of food and food ingredients would concur with these conclusions.

Joseph F. Borzelleca, Ph.D., F.A.T.S..
Chair of the Expert Panel
VCU School of Medicine
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Signature: _____
Date: 22 August 2008

Claire L. Kruger, Ph.D., DA.B.T.
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Medical Director
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Signature: _____
Date: 8/20/08

SUBMISSION END

AM

Spherix Consulting, Inc.

February 4, 2009

Moraima J. Ramos Valle
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3935

Dear Ms. Ramos-Valle:

Please find below the questions/clarifications requested by FDA and below these questions, our responses for each in **bold**.

Questions/Clarifications regarding GRN 268 - *Bifidobacterium longum* BB536:

1) Please revise Table I-1 and III-3 "Food Categories Proposed for Addition of *B. longum* BB536" (Page 2 and 34). Please refer to the bullets below.

   - The food categories proposed for addition of *B. longum* BB536 are listed in tables I-1 (page 2) and III-3 (page 34)*. Under the category of dairy products/dairy-based foods and dairy substitutes, "follow-on infant formula" (9th line) and "infant follow-on formula" (11th line) are listed, However, definition of these two terms (formulas) are not given in the tables/notification. Therefore, it is not clear what the differences are between these two terms (formulas). Please provide the definitions of these two terms (formulas).

     These two terms are the same and were mistakenly included twice in the food category list. For purposes of this GRAS dossier, we will change the nomenclature and use the term: Older Infant/Toddler Formula. This is a more precise description of the intended food product. These products are also known as follow-on baby formula and are designed for infants aged nine months and older who have started a diet supplemented by baby cereals and baby foods. (An example of this type of formula is Similac® Go & Grow Milk-Based Formula.) We have attached a revision of the Tables I-1 and III-3 with the terminology revised.

   - "follow-on infant formula" and "infant follow-on formula" are not included as part of the food categories listed under 21 CFR 107.3(n). Please revise table showing infant formula as a separate category.

     As discussed above, we have attached a revision of the tables with the terminology revised.

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- The titles and contents of these two tables appear to be identical except for the last line under miscellaneous. Please clarify.

  As discussed above, we have attached a revision of the tables with the terminology revised and the last line corrected.

2) Please clarify the specifications for the 5 product formulations. (Pg 16-21)

- BIFILON 50F is made with no milk ingredients, but has a milk protein specification.

  BIFILON 50F is produced to be a milk allergen free product, therefore, a specification for milk protein is included.

- BIFILON-50N, 50T and EX, have milk protein (high in EX), but are missing the milk specification.

  BIFILON 50N , 50T are not milk free products therefore no specification for milk protein is included.

3) Intakes and estimates are for 2 + years. Please clarify population that will be eating this ingredient.

- What age group this product is intended for? (follow-on infant formula could start at 9 months and "infant" is define as up to 12 months) .
- The age of infants for which each formula is intended should be provided.

  There are a variety of products that are intended for child/adult consumption. Intakes for age groups <12 mo, 12-23 mo, 2-5 y, 6-11 y, 12-18 y 19+ y and total population 2+ y are presented in Table III-4. The Older Infant/Toddler formula is intended for those infants greater than 9 months of age as described above.

4) Please comment on the use of dietary supplement usage in EDI discussion.

For completeness, a description of the use of bifidobacteria in dietary supplements was included in this chapter. Use of dietary supplements with probiotics was not included in the calculation of the EDI.

5) Page 57 of the notice reported on a chronic rat study (Momose, 1979) "Deaths in the control and test animals were unrelated to the administration of *B. longum* BB536 and the poor state of health of the surviving animals in this study makes interpretation of the results impossible. Therefore, it will not be described in further detail."

- Please report if there were any differences between the test and control group or not. If there is differences please report why it is or is not significant.
- Please provide an English translation of a copy of the Momose, 1979 article.

There were no differences that were determined to be toxicologically or biologically significant in this study. Deaths in both the control and test animals

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were due to respiratory infection unrelated to test article treatment, however, this resulted in difficulty interpreting this study. A copy of the Momose 1979 article is attached.

We have also attached a new manuscript which has been submitted for publication that was unavailable at the time of this GRAS submission. This new study "Safety evaluation of probiotic bifidobacteria by analysis of mucin degradation activity and translocation ability" supports the safety of BB536.

6) On page 51 of the notice, the notifier states that "results from the analysis indicate that there was no significant homology between the *B. longum* BB536 gene and the 10 selected genes from lactic acid bacteria with known antibiotic resistance… However, the notifier does not define what "significant homology" means. Please define significant homology.

The study to support this statement with definition of significant homology is attached.

7) On page 54 of the notice, the notifier states that "high homology between amino acid sequences in *B. longum* BB536 and the toxins and between amino acid sequences in *B. longum* BB536 and the known pathogens was not found. However, the notifier does not define what "high homology" means. Please define high homology.

The study to support this statement with definition of high homology is attached.

8) On page 53 in the "Summary of Antibiotic Resistance Testing", the notice states that "the strain is resistant to clinically important antibiotics…" Please clarify this statement.

This is a typographical error and should read "the strain is <u>not</u> resistant to clinically important antibiotics". A revision of page 53 with this correction made is attached.

If you have any additional questions or comments, please do not hesitate to contact me.

Best regards,

(b)(6)

Claire L. Kruger, Ph.D. D.A.B.T.
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Pages 000127-000128 under Freedom of Information exemption b(4) for trade secret.

3. Genetic Analysis for Known Toxins and Pathogenic Markers

The amino acid sequences of the predicted proteins derived from the *B.longum* BB536 genomic sequence were compared to the amino acid sequences of known bacterial toxins found in the Genebank database (release 152). The reference toxin sequences included those from *Clostridium botulinum*, *C.perfringens*, *C.difficile*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella thyphimurium*, *Escherichia coli*, *Shegella dysenteriae*, *Vibrio cholerae*, *C.tetani*, *Streprpcoccus pyogenes*, and several others (see Appendix 9 for complete list).

Additionally, gene sequences (as amino acid sequences) of three known pathogens were compared to gene sequences of *B.longum* BB536. The gene sequences from *Psedomonas aeruginosa* PA01(AE004091.1), *Staphylococcus aureus* N315(BA000018.3, AP003139.1), and *Clostridium perfringens* str.13(BA000016.3, AP003515.1) were used in the comparisons(see Appendix 9). Sequences were searched through the database of amino acid sequences expected from gene sequences of B.longum BB536 using BLASTP (amino acid vs. amino acid) and also using tBLASTN (amino acid vs. nucleic acid).

The homology between toxin or pathogenic genes and *B.longum* BB536 gene was evaluated in accordance with both "the expected value (E-value)" and "the sequence length rate for comparison in BLAST (SLR)". The sequence length rates (SLR) were obtained by below calculation formulas;
"Sequence length rate (query)" (%) = (HSP-gaps)/(query length) x 100
"Sequence length rate (subject)" (%) =(HSP-gaps)/(subject length) x 100

Morinaga defined homological level as below;
1) High homology level;
Expected value is less than 1e-70 and both sequence length rates for query and subject (*B. longum* BB536) are more than 98%.
2) Middle homology level:
Expected value is less than 1e-50 and both sequence length rates for query and subject (*B. longum* BB536) are more than 95%.
3) Low homology level;
Expected value is less than 1e-30 and both sequence length rates for query and subject (*B. longum* BB536) are more than 90%.

4) Very low homology level;

Expected value is less than 1e-10 and both sequence length rates for query and subject (*B. longum* BB536) are more than 80%

As homology analysis in accordance with BLASTP analysis and above definition, Morinaga obtained some genes defined any homology levels as shown in Table-1.

Table 1. Homology results between pathogenic or toxin genes and *B. longum* BB536 genes.

Query		E-value	SLR (query)	SLR (BB536)	Homology level
Acc	Species				
SA0102	*S. aureus*	0	105%	99%	High
CPE0378	*C. perfringens*	1e-172	104%	99%	High
PA0139	*P. aeroginosa*	6e-50	100%	100%	Middle
SA0366	*S. aureus*	1e-52	99%	101%	Middle
hlyB	*C. perfringens*	1e-39	93%	91%	Low
hlyA	*C. perfringens*	1e-46	97%	89%	Very low
hlyD	*C. perfringens*	5e-41	89%	86%	Very low
SA0657	*S. aureus*	4e-44	91%	89%	Very low

BLASTP results showed that *B.longum* BB536 had two high homological genes, SA0102 of *Staphylococcus aureus* (E-value = 0, SLR = 105% and 99%) and CPE0378 of *Clostridium perfringens* (E-value = 1e-172, 0, SLR= 104% and 99%). In case of SA0102, this protein is identified as hypothetical protein or myosin-cross reactive antigen in NCBI. However, in BLASTP analysis using NCBI, many traditional dairy lactic acid bacteria and the other bifidobacteria indicate high homology (low E-value) with SA0102. For example, the both E-value of *Lactococcus lactis* subsp. *cremoris* SK11, *Lactobacillus reuteri* F275, *L. reuteri* 100-23, *Lactococcus lactis* subsp. *lactis* Il1403, *Lactobacillus acidophilus* NCFM, *Lactobacillus gasseri* ATCC 33323, and *Bifidobacterium longum* DJO10A are all zero (0). The CPE0378 is also same with SA0102. When CPE0378 is analyzed homology by BLSTP in NCBI, many lactic acid bacteria indicate high homology (low E-value) with CPE0378. For example, the E-value of *B. adolescentis* ATCC 15703, *B. longum* DJO10A, *B. adolescentis* L2-32, *L. acidophilus* NCFM, *L. lactis* subsp. *cremoris* SK112e-172, *L. lactis* subsp. *cremoris* MG1363, *Lactobacillus johnsonii* NCC 533, *L. lactis* subsp. *lactis* Il1403, *L. gasseri* ATCC 33323, *L. reuteri* 100-23, and *L. reuteri* F275 are 9e-171, 1e-170, 3e-160, 3e-160, 5e-160, 3e-159, 1e-158, 4e-158, 2e-156, and 6e-156, respectively. Because there is no report indicating these lactic acid bacteria

have pathogen or toxicity, these proteins with high homology on SA0102 or CPE0378 are assumed to not be involved in pathogen or toxicity. Also, although both genes are identified as (probably) myosin-cross reactive antigen, this is not to say that the characteristic links pathogen or toxicity directory. From these results, Morinaga evaluated that the BB536 gene indicating high homology with both SA0102 and CPE0378 are not pathogenic and there is no problem for safety issue of *B. longum* BB536.

As a conclusion, Morinaga made a judgment that high homology between amino acid sequences in *B.longum* BB536 and the toxins and between amino acid sequences in *B.longum* BB536 and the known pathogens were not found in the gene analysis.

On the other hands, a few genes were applied to either middle, low, or very low homology levels in *B. longum* BB536 gene. PA0139 of *Pseudomonas aeruginosa* (E-value = 6e-50, sequence length rate = 100% and 100%) and SA0366 of *S. aureus* (E-value = 1e-52, sequence length rate = 99% and 101%) were applied to middle homology level as shown Table 1. Both PA0139 and SA0366 are annotated as ahpC (alkyl hydroperoxidereductase subunit C) in NCBI. And some scientists reported that ahpC was a gene related to reduction of oxygen toxicity and many aerobic bacteria had similar genes for prevention of oxygen toxicity. Also, it has been reported that *Bifidobacterium* species had oxygen metabolite enzymes like NADH-oxidase and NADH-peroxidase. So, it's no wonder that bifidobacteria had similar genes with ahpC for reduction of oxygen toxicity. Furthermore, when PA0139 and SA0366 were analyzed homology levels with the other bifidobacteria or lactic acid bacteria by BLASTP in NCBI, the similar homologies (E-value) with *B. longum* BB536 are observed. For example, in PA0139, the E-value of *L. lactis* subsp. *lactis* Il1403, *L. lactis* subsp. *cremoris* MG1363, *L. lactis* subsp. *cremoris* SK11, *B. longum* DJO10A, *B. longum* NCC2705, *L. casei* ATCC 334, *L. reuteri* F275, and *L. brevis* ATCC 367 were 7e-53, 1e-52, 2e-52, 7e-49, 9e-49, 1e-48, 2e-46, and 7e-46, respectively. In SA0366, the E-value of *L. lactis* subsp. *cremoris* MG1363, *L. lactis* subsp. *lactis* Il1403, *L. casei* ATCC 334, *L. reuteri* F275, *B. longum* DJO10A, *B. longum* NCC2705, and *L. brevis* ATCC 367 were 3e-72, 2e-71, 2e-54, 8e-52, 1e-51, 2e-51, and 4e-49, respectively.

From these observations and analysis, Morinaga evaluated that there is no problem for safety issue of *B. longum* BB536 even if *B. longum* BB536 have a gene which indicate a middle homology to both PA0139 and SA0366.

Also, BLATP analysis indicated that *B. longum* BB536 had low or very low

homology levels with some genes of Clostridium perfringens strain 13 which were annotated "probable hemolysin related protein". These genes were hlyA (E-value = 1e-46, sequence length rate = 97% and 89%), hlyB (E-value = 1e-39, sequence length rate = 93% and 91%), and hlyD (E-value = 5e-41, sequence length rate = 89% and 86%). However *B. longum* BB536 had no hemolysis activities on *in vitro* test and there has been no report that bifidobacteria has hemolytic activity before. And these hlyA, hlyB and hlyD of *C. perfringens* indicated similar homologies with many types of probiotic or dairy lactic acid bacteria in BLASTP analysis using NCBI. For example, in hlyA, the E-value between *Streptococcus thermophilus* CNRZ1066, *L. lactis* subsp. *lactis* Il1403, *L. lactis* subsp. *cremoris* MG1363, *Lactobacillus sakei* subsp. *sakei* 23K, *L. casei* ATCC 334, *B. adolescentis* L2-32, *B. adolescentis* ATCC 15703, *B. longum* DJO10A, *B. longum* NCC2705, *L. lactis* subsp. *lactis* Il1403, and *L. acidophilus* NCFM were 2e-53, 6e-53, 8e-53, 5e-51, 5e-47, 8e-46, 1e-45, 2e-45, 8e-43, 6e-38, and 3e-34, respectively. In hlyB, the E-value of *L. casei* ATCC 334, *L. lactis* subsp. *cremoris* SK11, *L. lactis* subsp. *lactis* Il1403, *La. sakei* subsp. *sakei* 23K, *St. thermophilus* CNRZ1066, *L. lactis* subsp. *lactis* Il1403, *Lactobacillus salivarius* subsp. *salivarius* UCC118, *B. adolescentis* ATCC 15703, *B. longum* DJO10A, and *L. acidophilus* NCFM are 1e-68, 5e-65, 5e-64, 1e-63, 1e-62, 9e-45, 1e-40, 3e-39, 3e-38, and 4e-36 respectively. In hlyD, the E-value for *L. plantarum* WCFS1, *L. casei* ATCC 334, *L. sakei* subsp. *sakei* 23K, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, *L. reuteri* 100-23, *L. reuteri* F275, *S. thermophilus* LMG 18311, *L. acidophilus* NCFM, *L. lactis* subsp. *cremoris* MG1363, *L. lactis* subsp. *cremoris* SK11, *L. johnsonii* NCC 533, *L. gasseri* ATCC 33323, and *B. longum* NCC2705 are 3e-72, 6e-72, 4e-71, 8e-71, 2e-70, 7e-70, 9e-70, 3e-68, 1e-67, 2e-67, 5e-66, 2e-65, and 8e-40, respectively. Because most of dairy lactic acid bacteria have no hemolysis activity, these genes of lactic acid bacteria were not assumed to related to hemolysin directly. These findings suggested that the *B. longum* BB536 genes indicating low or very low homology levels with "probable hemolysin" genes of *C. perfringens* were no problem for the safety issues of *B. longum* BB536.

SA0657 of *S. aureus* is, also, indicated very low homology level (E-value = 4e-44, sequence length rate = 91% and 89%). Although this gene is defined as hypothetical protein in NCBI, there are some comments that this protein might related with hlyC. However, the SA0657 of *S. aureus* indicated similar homologies with many types of probiotic or dairy lactic acid bacteria in BLASTP analysis using NCBI. For example, the E-value of *L. lactis* subsp. lactis Il1403, *L. reuteri* F275, *L. reuteri* 100-23, *L. brevis* ATCC 367, *L. sakei* subsp. *sakei* 23K, *L. casei* ATCC 334, *L. gasseri* ATCC 33323, *L. acidophilus* NCFM, *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365, *L plantarum*

WCFS1, *L. reuteri* 100-23, *L. lactis* subsp. *cremoris* SK11, *S.thermophilus* CNRZ1066, *L. lactis* subsp. *lactis* Il1403, *L. johnsonii* NCC 533, and B. *longum* NCC2705 were 2e-78, 4e-66, 9e-66, 7e-64, 1e-63, 7e-63, 1e-62, 6e-62, 9e-61, 4e-58, 1e-54, 1e-50, 2e-50, 9e-50, 8e-49, and 5e-40, respectively. These findings and the facts that *B. longum* BB536 has no homolysis activity indicated that the gene of BB536 indicating very low homology with SA0657 was no problem for BB536 safety issues.

Furthermore, E-value of BLASTP analysis involved three rpoB genes of *C.perfringens*, *S.aureus*, and *P. aeruginosa* were zero (0) although the sequence length rates were lower than very low homology level. The rpo B gene is generally annotated as RNA polymerase subunit and is conserved very well in many kinds of bacteria including lactic acid bacteria. The E-values of many kinds of lactic acid bacteria were almost zero (0). These facts indicated that these rpoB gene of lactic acid bacteria were not involved in pathogen and that the presence of a gene indicating similarity with rpoB in *B. longum* BB536 was no problem for safety.

From above comprehensive and rigorous analysis, Morinaga evaluated that *B. longum* BB536 had no gene related to toxin and pathogen.

Pages 000134-000141 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Corrected pages for:

Generally Recognized As Safe (GRAS) Notification for the Use of *Bifidobacterium longum* BB536 in Selected Foods

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Table I-1. Food Categories Proposed for Addition of *B. longum* BB536

Breads/baked goods	
	bars; includes meal replacements, high protein, snack bars
	biscuits
	breads/rolls (yeast); includes bagels, croissants, English muffins, pizza crust
	breakfast pastries; includes Danish
	cakes, includes coffee cakes
	cobblers, turnovers, strudels, crisps
	cookies/bars
	crackers
	doughnuts
	pies
	quick breads; includes breads, muffins, popovers, cornbread
Cereals	
	breakfast cereals, cooked; includes grits, oatmeal, cream of wheat, wheat cereal
	breakfast cereals, ready-to-eat
Dairy products/dairy-based foods and dairy substitutes	
	cheese spreads
	cheese, imitation
	cheese, processed
	cream substitutes
	cream, heavy
	fermented milk; includes buttermilk and kefir
	flavored milk beverage mixes
	frozen desserts; includes ice cream, ice milk, frozen yogurt, frozen novelties, milk shakes
	older Infant/Toddler formula
	imitation milk
	meal replacements, liquids and dry mixes
	milk, plain and flavored; includes cocoa, chocolate milk, fruit milks, coffee drinks (fluid/dry)
	puddings and custards
	smoothies
	whipped toppings
	yogurt
Fruit Products	
	juices and nectars; includes citrus, non-citrus, vegetable and blends
	frozen fruit
	frozen juice bars, ices
Miscellaneous	
	candies; includes hard candies, mints, chocolate, and all other types of confections
	chewing gum
	cocoa powder
	condiment sauces, including catsup, BBQ, taco, steak, cocktail, Worcestershire, teriyaki, tartar
	flavored beverage syrups
	fruit flavored powder beverage mixes
	gelatin desserts, plain or with fruit
	gravies
	margarine
	peanut and other nut butters/spreads
	snack foods; including chips, popcorn mixtures
	weaning foods (for children 12 months of age and older; includes dry cereal, snacks, juices)

Table III-3. Food Categories Proposed for Addition of *B. longum* BB536

Breads/baked goods
- bars; includes meal replacements, high protein, snack bars
- biscuits
- breads/rolls (yeast); includes bagels, croissants, English muffins, pizza crust
- breakfast pastries; includes Danish
- cakes, includes coffee cakes
- cobblers, turnovers, strudels, crisps
- cookies/bars
- crackers
- doughnuts
- pies
- quick breads; includes breads, muffins, popovers, cornbread

Cereals
- breakfast cereals, cooked; includes grits, oatmeal, cream of wheat, wheat cereal
- breakfast cereals, ready-to-eat

Dairy products/dairy-based foods and dairy substitutes
- cheese spreads
- cheese, imitation
- cheese, processed
- cream substitutes
- cream, heavy
- fermented milk; includes buttermilk and kefir
- flavored milk beverage mixes
- frozen desserts; includes ice cream, ice milk, frozen yogurt, frozen novelties, milk shakes
- older Infant/Toddler formula
- imitation milk
- meal replacements, liquids and dry mixes
- milk, plain and flavored; includes cocoa, chocolate milk, fruit milks, coffee drinks (fluid/dry)
- puddings and custards
- smoothies
- whipped toppings
- yogurt

Fruit Products
- juices and nectars; includes citrus, non-citrus, vegetable and blends
- frozen fruit
- frozen juice bars, ices

Miscellaneous
- candies; includes hard candies, mints, chocolate, and all other types of confections
- chewing gum
- cocoa powder
- condiment sauces, including catsup, BBQ, taco, steak, cocktail, Worcestershire, teriyaki, tartar
- flavored beverage syrups
- fruit flavored powder beverage mixes
- gelatin desserts, plain or with fruit
- gravies
- margarine
- peanut and other nut butters/spreads
- snack foods; including chips, popcorn mixtures
- weaning foods (for children 12 months of age and older; includes dry cereal, snacks, juices)

d. Summary of Antibiotic Resistance Testing

In summary, the available information on antibiotic resistance patterns of *B. longum* BB536 indicates that the antibiotic susceptibilities of the strain are overall similar to patterns of other bifidobacterial species, the strain is not resistant to clinically important antibiotics, and the strain is not likely to have transmissible antibiotic genes. These findings indicate that use of *B. longum* BB536 in foods does not present concerns for antibiotic resistance.

2. Metabolic Activities

a. D-Lactate Production

Metabolic products of both *Bifidobacterium longum* BB536 and *B. longum* ATCC 15707 (type strain) were cultured and analyzed for the presence of both D- and L- lactic acids (Table V-4). Results from this study indicate that *B. longum* BB536 produces predominantly L-lactic acid, while production of D-lactic acid is negligible.

Table V-5. Lactic Acid Production by *B. longum* BB536 and *B. longum* ATCC 15707

Strain	D-Lactic acid (mg/g)	L-Lactic acid (mg/g)	Total (mg/g)
B. longum BB536	0.07	4.00	4.07
B. longum ATCC 15707[a]	-0.05	5.67	5.67

Notes:
Source Morinaga Milk Co., Ltd Industry
Abbreviations:
[a] Type strain

b. Bile Salt Deconjugation

Grill and colleagues conducted studies (1995a and 1995b) to investigate bile salt deconjugation by bifidobacteria. In one study, Grill et al. (1995a) investigated the effect of bile salt concentrations on selected species of bifidobacteria, including *B. longum* BB536. At bile salt concentrations ranging from 0.95 to 5.5 mM, strong and rapid inhibition of growth (>80%) was observed for all bifidobacteria species, and at concentrations above 5.5 mM, complete inhibition of bacterial growth was seen. *B. longum* BB536 was observed to deconjugate 80-95% of selected bile salts. The production of deconjugated bile salts was concurrent with bacterial growth, and deconjugated bile salts were the only compound produced during bifidobacteria transformation. The investigators attributed the growth inhibition of the bile salts to production of deconjugated

Reference List for Industry Submission, GRN 000268

Pages	*Author*	*Title*	*Publish Date*	*Source*	*BIB_Info*
000134-000141	Momose, H; Igarashi, M.; Era, T.; Fukuda, Y.;Yamada, M.; Ogasa, K.	Toxicological studies on Bifidobacterium longum BB-536	1979	Pharmacometrics	Volume 17, pgs 881-887

NA- Not applicable